Proceedings of the Seventh Canadian Workshop on Harmful Marine Algae

J.N.C. Whyte (Editor)

Fisheries and Oceans Canada
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by

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ABSTRACT

Whyte, J.N.C. (*Editor*). 2001. Proceedings of the seventh Canadian workshop on harmful marine algae. Can. Tech. Rep. Fish. Aquat. Sci. 2386: xiii + 158 p.

The Seventh Canadian Workshop on Harmful Marine Algae, sponsored by Fisheries and Oceans Canada, Pacific Region, was held in Nanaimo, British Columbia, on May 23-25, 2001. Ninety-nine participants from Canada and nine other countries attended the workshop as representatives of research and management departments within federal and provincial governments, universities, and the private sector. The thirty five oral and twenty six poster presentations encompassed current knowledge and awareness of Harmful Algal Blooms (HABs). Topics included: HABs impacting fisheries and management strategies; ecological factors influencing HAB formation and distribution; monitoring, protocols, occurrence and distribution of HABs; emerging assays for marine biotoxins; public health issues and phycotoxins management; analysis, effects, and mechanisms for production of biotoxins; interactions of HAB species with other organisms; and spatial distribution of phycotoxins and kinetics of animal toxicity. Contained in this report are abstracts, extended abstracts, and papers of the oral and poster presentations, together with reports from two Working Group discussions. The subjects of the Working Groups were: HAB interactions affecting fisheries and potential protocols for mitigation of HAB effects; and phytoplankton monitoring related to toxin distribution, and innovations and application of biotoxin assessment related to public health issues.

RÉSUMÉ

Whyte, J.N.C. (*Editor*). 2001. Proceedings of the seventh Canadian workshop on harmful marine algae. Can. Tech. Rep. Fish. Aquat. Sci. 2386: xiii + 158 p.

Le Septième atelier de travail Canadien sur les algues marines nuisibles, s'est tenu du 23 au 25 mai 2001sous l'égide de Pêches et Océans Canada, région du Pacifique, à Nanaimo, Colombie Britannique. Quatre vingt dix neuf participants du Canada et neuf participants d'autres pays étaient présents et représentaient la recherche et la gestion effectués par les différents niveaux de gouvernement provincial et fédéral, dans les universités ainsi que dans le secteur privé. Trente cinq communications et vingt six affiches illustrant l'état des connaissances sur les floraisons d'algues nuisibles (FAN) ont été présentés. Les sujets étaient : FAN ayant un impact sur les pêcheries et leurs gestions; les facteurs écologiques influencant la formation et la distribution des FAN; la surveillance, les protocoles déchantillonnage, l'abondance et distribution des FAN; les nouvelles méthodes de dosages des biotoxines marines; la santé publique et la gestion des phycotoxines; l'analyse, les effets et les mécanismes de production des biotoxines; l'interaction des espèces productrices de toxines avec les autres organismes ainsi que la distribution spatiale des phycotoxines et la cinétique de toxicité animal. Dans ce rapport on trouvera les résumés des communications, les abrégés des présentations orales et affichées ainsi que le comptes rendus des deux groupes de discussion. Les sujets abordés par les groupes de discussions ont été l'interaction des algues nuisibles avec les pêcheries et les mesures étudiées pour en atténuer les effets; la surveillance du phytoplancton en relation avec à la distribution des toxines; l'évaluation des innovations et des application des essais sur biotoxines en relation avec la santé publique.

ACKNOWLEDGEMENTS

The Seventh Canadian Workshop on Harmful Marine Algae was sponsored by the Science Branch, Pacific Region, of Fisheries and Oceans Canada. The welcoming address by Dr. Laura Richards, Regional Director of Science, was very much appreciated. Members of the Phycotoxins Working Group, particularly Jennifer Martin, are thanked for their advice on planning the workshop. The success of the meeting was in large measure due to the efficient fulfilment of logistical tasks undertaken by the local advisory committee consisting of Nicky Haigh, Norma Ginther, Laurie Keddy, Ann Thompson, and Linda Hiemstra. Thanks to student volunteers from Malaspina University College who gave so generously of their time during registration. Special thanks to students Alicia Hooper and Jesse Ketler for their proficient handling of the audio-visual systems. I am indebted to Linda Hiemstra and Susan Farrant from Malaspina University College for their assistance in co-ordinating the sale of tickets and financial planning of the workshop banquet. Finally, my thanks to Lyse Godbout of the Pacific Biological Station for the French translation of the workshop announcements and abstract.

INTRODUCTION

Production of biotoxins and the physical damage caused by marine phytoplankton have become crucial issues concerning public health and fisheries resources world-wide. In Canada the impact on human health from consumption of toxic shellfish has been recognized since recorded time on both the Pacific and Atlantic coasts. Finfish mortality caused by Harmful Algal Blooms (HABs) has become more prevalent with the advent and expansion of finfish aquaculture. The extent of economic losses to persistent blooms is evident from the $95.5 million loss to Korean fisheries in 1995, the recent $32 million loss in aquacultured finfish in Hong Kong in 1998, and to a lesser extent the cumulative $35 million loss in aquacultured salmonids in British Columbia since the mid 1980's. These economic losses seriously impact the socio-economic structure of coastal communities. Although these harmful algae have a profound economic impact on shellfish and aquacultured finfish, little is known to date of their harmful effects on recruitment of wild fisheries.

In British Columbia today, Paralytic Shellfish Poisoning continues to be a hazard to public health and safety. The toxicity in shellfish originating from species of *Alexandrium* has been monitored routinely since the mid 1960's to ensure the safety of shellfish as a food product. Another human health risk related to consumption of shellfish harvested on this coast is Amnesic Shellfish Poisoning, caused by domoic acid from species of the diatom *Pseudo-nitzschia*. These risks have been minimized through regular monitoring of the toxicity levels in commercial bivalves by the Canadian Food Inspection Agency.

Following the deaths and illnesses of people caused by the consumption of domoic acid contaminated mussels from Prince Edward Island in 1987, Fisheries and Oceans Canada established the Phycotoxins Working Group. This advisory committee set out to foster scientific research and to encourage exchange of scientific information on harmful and toxic algae. Other objectives were to monitor and understand the biology and ecological significance of bloom formation, to determine the distribution of algal toxins in the ecosystem, to foster the development of new toxin bioassays, and to evaluate the effects of harmful species on other marine organisms. This knowledge was required to develop protocols for mitigation of the adverse effects on human health and economic losses to wild and aquacultured fisheries.

Exchange of knowledge and scientific discussion has been further advanced through the establishment of national workshops on harmful marine algae, organized by the Phycotoxins Working Group and sponsored by Fisheries and Oceans Canada. The first Canadian Workshop was held in Moncton, New Brunswick in 1989 and five more have been held since then at different centres on the Atlantic or Pacific coasts. This year, 2001, the Pacific Region hosted the Seventh Canadian Workshop on Harmful Marine Algae in Nanaimo.

HABs are natural phenomena that appear to be increasing in frequency throughout the world. Only through increased knowledge and understanding of the damage caused by harmful algae will we be able to fully appreciate their impact on the marine environment and manage fisheries resources appropriately. This workshop provided a forum for the presentation and discussion of current knowledge of the many and varied aspects of harmful algae.

Previous Workshop Proceedings in this Series and Related Phycotoxins Working Group Activities

Martin, J.L. and K. Haya (*Editors*). 1999. Proceedings of the Sixth Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 2261: x + 159 p.

Penney, R.W. (*Editor*). 1996. Proceedings of the Fifth Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 2138: xiii + 195 p.

Bates, S.S. and P.D. Keizer (*Editors*). 1996. Proceedings of the Workshop on Harmful Algae Research in the DFO Maritimes Region. Can. Tech. Rep. Fish. Aquat. Sci. 2128: v + 44 p.

Forbes, J.R. (*Editor*). 1994. Proceedings of the Fourth Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 2016: viii + 92 p.

Therriault, J.-C. and M. Levasseur (*Editors*). 1992. Proceedings of the Third Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 1893: iv + 154 p.

Gordon, D.C., Jr. (*Editor*). 1991. Proceedings of the Second Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 1799: iv + 66 p.

Forbes, J.R. (*Editor*). 1991. Pacific Coast Research on Toxic Marine Algae. Can. Tech. Rep. Hydrogr. Ocean Sci. 135: vi + 76 p.

Bates, S.S. and J. Worms (*Editors*). 1989. Proceedings of the First Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 1712: iv + 57 p.

ORAL SESSION 1:

HABs Impacting Fisheries and Management Strategies

THE TOXIC DINOFLAGELLATE *ALEXANDRIUM TAMARENSE* ASSOCIATED WITH MORTALITY OF FARMED SALMON IN NOVA SCOTIA

Allan D. Cembella*[1], Michael A. Quilliam[1], Nancy I. Lewis[1], Andrew G. Bauder[1], Krista Thomas[1], Joanne F. Jellett[2], Hugh Ferguson[3], Claire Carver[4] and Roland R. Cusak[3]

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In early June (2000), elevated fish mortalities (ca. 2000 individuals) were reported in cages of Atlantic salmon (*Salmo salar* L.) from the southeast coast of Nova Scotia. Dead fish exhibited severe edema of the gills accompanied by mucus production. There was no evidence of infectious aetiology in post-mortem analysis. The water column adjacent to the cages was characterized by a sharp pycnocline, after salinity-dependent stratification due to abundant freshwater runoff. *In situ* fluorescence profiles revealed a high sub-surface (2 to 4 m depth) chlorophyll peak related to a phytoplankton bloom. Microscopic analysis of plankton collected near the cages during and subsequent to the fish mortality event showed abnormally high concentrations (max. 8×10^5 cells l^{-1}) of the marine dinoflagellate *Alexandrium tamarense*, a known producer of potent neurotoxins associated with paralytic shellfish poisoning (PSP). This intense bloom was virtually monospecific (>98% of the >20 μm net plankton), and the high PSP toxin content of plankton size-fractions was directly related to *Alexandrium* cell concentration. The toxin profile of the 21-56 μm plankton size-fraction was complex, dominated by the N-sulfo-carbamoyl derivative C2, with lesser amounts of gonyautoxins (GTX) 1,3,4, neo-saxitoxin (NEO), and saxitoxin (STX), and a trace of decarbamoyl-GTX. Cultured clonal isolates of *A. tamarense* from this site were very toxic (3-60 pg STXequiv./cell), and yielded diverse toxin spectra. No PSP toxins were detected in samples of salmon liver, muscle or digestive tract by analysis using LC-MS and LC with fluorescence detection, but a sensitive immunoassay test (Jellett Biotek) revealed a low level (mean: *ca.* 10 ng STXequiv./g; n = 3) of these toxins in gills. Given the high ambient concentration of *Alexandrium* and entrapment of these cells in mucus of gill lamellae, the fish mortalities were likely caused by direct exposure to toxic cells and/or to soluble toxins released during the bloom.

MANAGING A POPULAR RECREATIONAL SHELLFISH FISHERY AROUND HARMFUL ALGAL BLOOMS

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Montesano, WA 98563 USA.

Perfect habitat for the Pacific Razor Clam (*Siliqua patula*, Dixon) is found along the Pacific Ocean beaches in Washington State. The State has managed these razor clam populations since the early 1900's. What had became a large commercial fishery by the 1940's, evolved into a major recreational fishery, peaking in the late 1970's with an annual harvest of more than 15 million clams during one million digger trips. The economies of many small coastal communities depend heavily on the visitors who travel long distances to harvest razor clams. In the decades preceding the 1990's, marine toxins were not a major issue. Unsafe levels of PSP were never detected in routine tests performed by the Washington Department of Health (WDOH). However in November 1991, high levels of domoic acid were detected in razor clam tissue and the recreational fishery was closed immediately. High levels of domoic acid have now disrupted this popular recreational fishery five times - twice for more than a year. WDOH now requires extensive pre- and in-season sampling. This process often allows only one or two days to notify thousands of harvesters that the season has been cancelled. This has disrupted local economies and has required Washington Department of Fish and Wildlife (WDFW) enforcement officers to set up and staff roadblocks. Currently, WDFW is actively participating in the federally funded Olympic Region Harmful Algal Bloom (ORHAB) project. This study provides the hope of developing an "early-warning" system to avoid the disruptions caused by last-minute season fishery closures, while still protecting the health of thousands of clam diggers.

INFLUENCE OF *COCHLODINIUM POLYKRIKOIDES* BLOOMS ON AQUACULTURED SALMONIDS

J.N.C. (Ian) Whyte*, Nicola Haigh, Norma G. Ginther and Laurie J. Keddy

Fisheries and Oceans Canada, Pacific Biological Station, 3190 Hammond Bay Road, Nanaimo, BC, Canada V9R 5K6

Detailed phytoplankton monitoring of aquaculture sites on the west coast of Vancouver Island identified for the first time the formation of *Cochlodinium polykrikoides* blooms that lasted from August to October 1999. In Quatsino Sound blooms migrated eastwards as the season progressed to adversely affect farmed salmon in Holberg Inlet (Fig. 1).

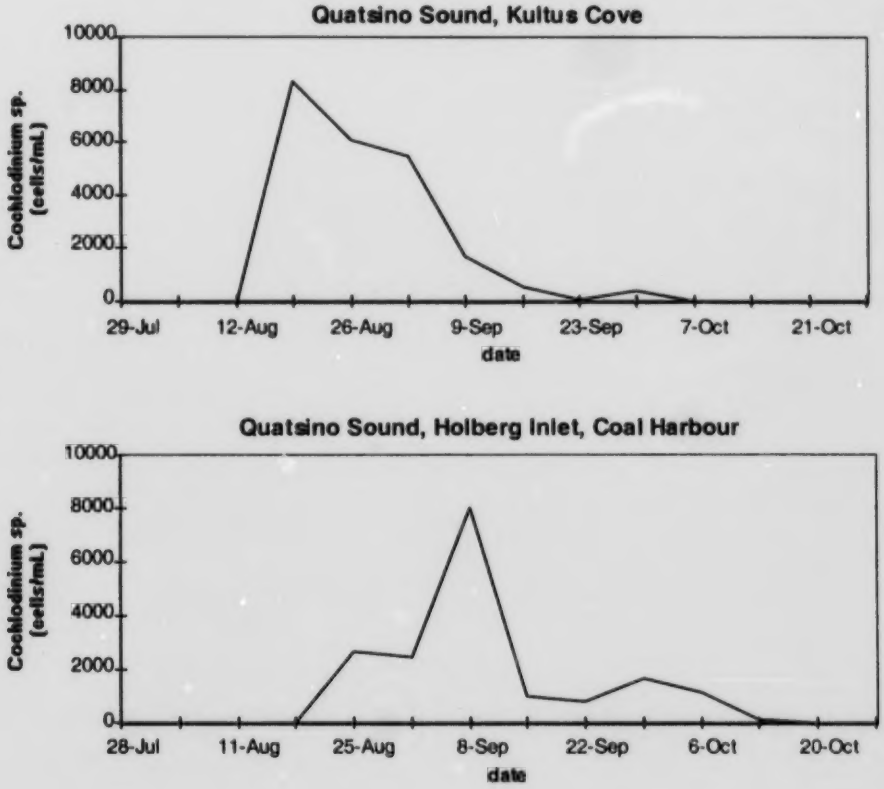

Fig. 1. *Cochlodinium polykrikoides* concentrations at Kultus Cove (50° 29' N - 127° 43' W) and Coal Harbour (50° 36' N - 127° 35' W) from August to October 1999.

Farmed Atlantic salmon, *Salmo salar*, were observed to stop feeding when exposed to 500 cells mL^{-1} and mortality occurred above 2000 cells mL^{-1}. Bioassays conducted in the inlet with Atlantic salmon smolts indicated that percentage mortality was a function of cell density. This was corroborated under controlled laboratory conditions when exposure of Atlantic salmon to the bloom caused 100% mortality when exposed 27 min to 7,200 cells mL^{-1}, 55 min to 3,400 cells mL^{-1}, and although fish appeared distressed in 1,000 cells mL^{-1} only 20% died within the 24 h bioassay. By contrast 100% mortality of Pacific coho salmon, *Oncorhynchus kisutch*, occurred only after 50 and 200 min exposure to 10,700 and 9,600 cells mL^{-1}, suggesting species-specific susceptibility of salmonids to the effects of *Cochlodinium*. Enhanced toxicity of *Cochlodinium* observed when the dinoflagellate was oxygenated or aerated may have been a function of agitation leading to increased release of lethal reactive oxygen species or increased release of algal mucus, which is known to smother benthic organisms. The mucus, most likely a glycoprotein, produces a thick gel pellet encasing algal cells when the alga is centrifuged. Once formed this pellet resists ready dissolution. Corroboration of a high content of carbohydrate mucus in the alga is verified by proximate analysis (Fig. 2), which indicates 18.6% polysaccharide and 4.5% mono- and oligo-saccharides.

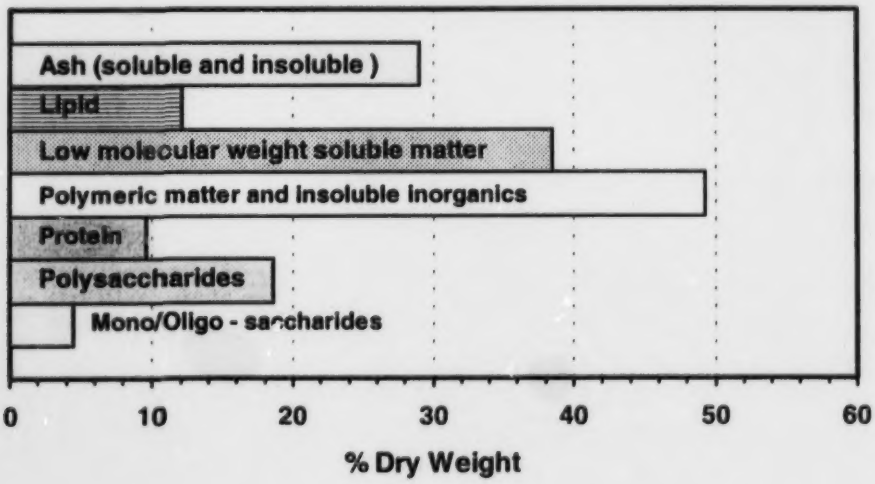

Fig. 2. Proximate analysis of *Cochlodinium polykrikoides* from the bloom at Coal Harbour

The strong diurnal pattern, with high cell concentrations overnight at depths to 25 m and at the surface during the day, made mitigation protocols using 14 m deep tarps and upwelling of deep water by aeration less effective than for *Heterosigma akashiwo*. Phytoplankton data from farm sites around Vancouver Island in 1999 and 2000 tends to suggest an increasing distribution of ichthyotoxic *Cochlodinium polykrikoides* on the west coast of Canada (Figs. 3 and 4).

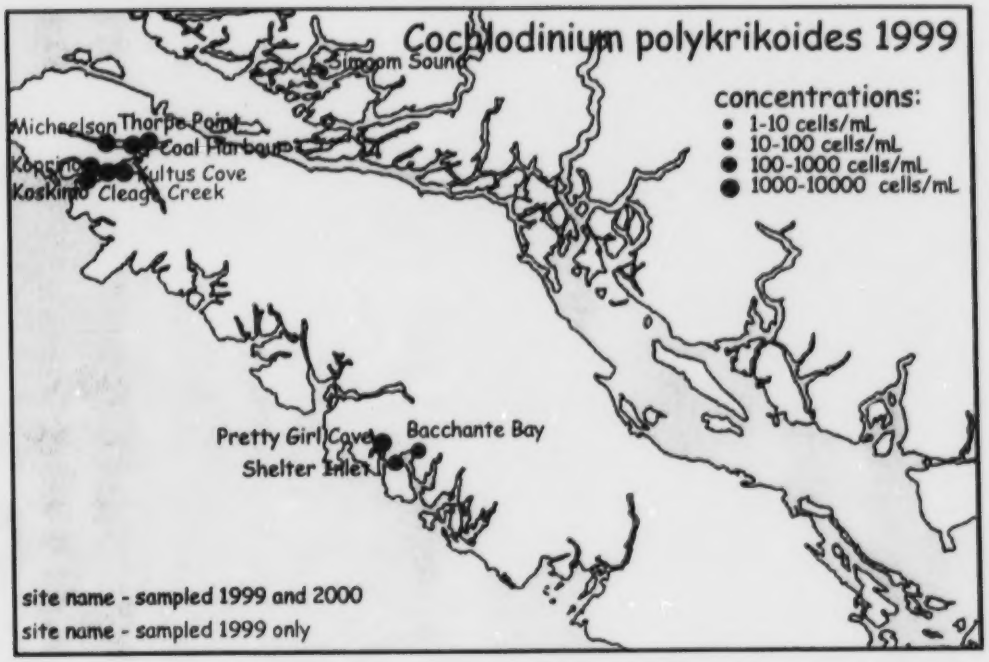

Fig. 3. Distribution and concentration of *Cochlodinium polykrikoides* in 1999

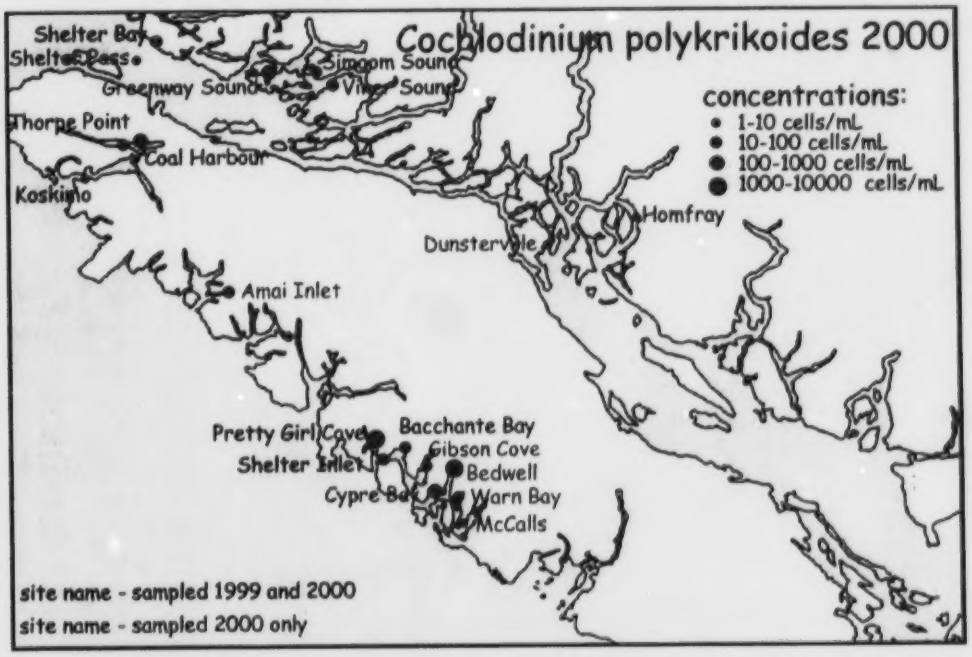

Fig. 4. Distribution and concentration of *Cochlodinium polykrikoides* in 2000.

ORAL SESSION 2:

Ecological Factors Influencing HAB Formation and Distribution

THE ROLE OF PRECIPITATION AND RIVER RUN-OFF ON *ALEXANDRIUM TAMARENSE* BLOOMS IN THE GULF OF ST. LAWRENCE

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Blooms of the toxic dinoflagellate *Alexandrium tamarense*, a causative organism of paralytic shellfish poisoning (PSP), are annually recurrent events in the Gulf of St. Lawrence, Québec, Canada. The data analysis of harmful algal blooms between mid-May and mid-October from 1989 to 1998 at Sept-Îles, a presumed initiation site in the Gulf of St. Lawrence, revealed great yearly fluctuations in the onset, duration, and magnitude of toxic *A. tamarense* blooms. The analysis of the results combined with the hydrological and meteorological data for the region indicate that the interactions between precipitation, local river run-off and the wind regime greatly affect the pattern of bloom development each year. Results from the 10-year data set reveal that in this system: 1) the probability of observing *A. tamarense* cells in the water column increases with decreasing salinity; 2) strong summer rainfall events can initiate *A. tamarense* blooms; and 3) periods of high local river run-off combined with low wind speeds favor the development of *A. tamarense* blooms. Salinity, which reflects the general state of the water column in terms of freshwater input and stability, could thus be used as a predictive tool at this station.

OCEANOGRAPHIC AND ENVIRONMENTAL ASSESSMENT OF KUWAIT BAY IN RELEVANCE TO TOXIC ALGAL BLOOMS

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Globally, the frequency of the incidence of red tides has been on the increase. Some of the algal bloom incidences are attributed to urban pollution and eutrophication. This is true in the waters off Kuwait where 21 potentially harmful algal species exist. The latest to the list are the suspects *Gymnodinium selliforme* (?) and *Prorocentrum mexicanum*, implicated in the massive mortality of about 150 tons of culture sea bream and 30 tons of wild mullets during September-October 1999. Because of the economic impact, i.e., loss of revenue, and societal impact, a one-year study was conducted to investigate the red tides in Kuwait Bay. Objectives of this project include quantitative studies of phytoplankton in Kuwait Bay waters in close proximity to sewage outfalls and testing the hypothesis that eutrophication is initiating red tides in Kuwait Bay. Phytoplankton biomass at the stations off the sewage outfalls was higher than the biomass at the station away from the outfall. In addition to the field surveys, a perturbation experiment was conducted which demonstrated that natural assemblages of phytoplankton from the Kuwait Bay environment could be stimulated and sustained to bloom levels. High levels of algal density (up to 28.9 million flagellates/L, 31.6 million picoplankters/L and 8.9 million other algal cells/L) were attained. Phytoplankton biomass measured in terms of chlorophyll also responded similarly. From this study, it is evident that enrichment with sewage could induce development of algal blooms in microcosms. The magnitude of the blooms and their production characteristics were high and comparable to red tide waters off this coast. The presence of a variety of phytoplankton in bloom densities, particularly the dinoflagellates implicated in toxigenic episodes elsewhere is a matter of concern and demonstrated the existence of a potential for development of toxigenic algal blooms off this coast due to eutrophication.

THE INFLUENCE OF SEASONAL MONSOONS ON THE OCCURRENCE OF HABs IN HONG KONG WATERS

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Red tides have frequently occurred in Hong Kong waters over the past 20 years. There have been a few hundred red tide cases during the period from 1983-1998. An analysis of these cases reveals that there was a spatial-temporal progression of red tides. Red tides during winter and early spring occurred in the northeast and spread to the west during later spring and summer. Most red tides (74%) occurred in sheltered waters (the northeastern waters) such as Tolo Harbour, Mirs Bay and Porter Shelter to the east of Hong Kong and 70% of the total red tide cases occurred between December and May and the lowest frequency (<5%) was during July-November. These spatial and temporal patterns appear to be related to the seasonal monsoons. The northeast winds prevail in winter and spring, causing downwelling against the shore, or an inflow towards semi-enclosed waters along the shore. As a result, the flushing rate of these semi-closed waters in Hong Kong is reduced and local conditions like shallow waters and a high input of nutrients become dominant and favorable to phytoplankton blooms. In summer, usually the southwest winds blow and result in outflow from these semi-enclosed waters. Rainfall is also maximal in summer. The addition of freshwater from rainfall decreases salinity and increases the outflow rate in these semi-enclosed waters. As a result, the deep oceanic waters that are nutrient poor on the continental shelf are drawn into the bays. The increased flushing rate, the sudden decrease in salinity and the entrainment of nutrient poor bottom water do not favor red tide blooms.

THE 1998 RED TIDE OF *ALEXANDRIUM TAMARENSE* IN THE LOWER ST. LAWRENCE ESTUARY (CANADA): IMPORTANCE OF CLIMATIC CONDITIONS AND PHYSICAL TRANSPORT

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In 1998, a red tide developed in the Lower St. Lawrence Estuary (LSLE). The causative organism was *Alexandrium tamarense*, a dinoflagellate responsible for PSP outbreaks in the LSLE. In order to estimate the role of climatic factors and advection on the onset of the red tide, the temporal distribution of *A. tamarense* in the LSLE was compared with the prevailing climatic conditions (rain, wind, tides, water temperature, cloud cover) and the retention of surface water during the summer 1998 in the LSLE. A three-dimensional circulation model, the regional ocean model (ROM) of the Gulf of St. Lawrence, is used to determine the retention times in the outbreak area. Our results show that the onset of the 1998 red tide coincided with a period of increased stratification and followed a period of dominant north-easterly winds resulting in the retention of surface water which allowed the growth and accumulation of *A. tamarense* cells in the estuary. To estimate the importance of physical transport on the development of *A. tamarense* blooms, eulerian passive tracers were incorporated in the ROM to simulate the transport of the bloom in the estuary. The model was initialized with observations of *A. tamarense* abundance in the LSLE during the pre-bloom period of 1998 and the results of the simulations were compared with *A. tamarense* distributions observed during the bloom and post-bloom periods.

TEMPERATURE AND HARMFUL ALGAE IN AYSEN FJORD (45°26´S 73°00´W), CHILE, BETWEEN 1993 AND 1999

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Blooms of DSP and PSP producing micro algae, and several taxa related to damages to caged salmon have been detected since 1991 in Aysen fjord (45°26´S 73°00´W), but have increased in intensity and frequency in recent years. The outbreaks of *Alexandrium catenella* and *Dinophysis* species present in the fjord were recorded from 1993 till 1999, on a 6-year time series based on qualitative phytoplankton samples and superficial temperature measurements taken every 15 d. This was the first study to use such a long time series for knowing the dynamics and temporal variability of the phytoplankton in the area. During the analysis, a total of 116 species and varieties were identified: 84 corresponding to diatoms, 30 to dinoflagellates and 2 to silicoflagellates, being from marine neritical and oceanic, estuarine, continental and benthic habitats. Between 1993 and 1999, 34 blooms of *Dinophysis acuta* and 45 blooms of *Dinophysis acuminata* were detected, while *Alexandrium catenella* was present only 8 times in the samples. All the toxin related algae were present on March 1998 in one of the biggest algae blooms recorded, when 6 people died from PSP and DSP combined intoxication. The superficial water temperature presented normal annual fluctuations for a cold temperate region, with extreme values of 5.2° and 16.2°C, with the lowest being present during 1996 and the highest in 1994 and 1998, which were years that presented ENSO phenomena. The warmer years presented higher presence of toxic species, while the species harmful to the salmonid culture appeared with estuarine conditions and cryophilic species. Scattering analysis determined an optimal temperature range between 10.5° C and 15.5° C for the three toxins related species further explaining the mixed blooms detected on March 1998.

ORAL SESSION 3:

Monitoring, Protocols, Occurrence and Distribution of HABs

ANNUAL TIMING OF PHYTOPLANKTON BLOOMS IN THE WESTERN BAY OF FUNDY

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As part of a regional toxic phytoplankton-monitoring program, phytoplankton has been monitored since 1988 at a fixed station near the Wolves Islands in the western Bay of Fundy. Samples have been collected at four depths (0, 10, 25 and 50 m) at weekly to monthly intervals using water-sampling bottles. Water samples for phytoplankton enumeration were settled in Zeiss counting chambers and all phytoplankton greater than 5 µm were identified and enumerated using a Nikon inverted microscope. Further identification was done using either a JEOL JSM-5600 scanning electron microscope or an Hitachi S-2400 scanning electron microscope. We have begun to explore this data set from several perspectives, including that of the annual timing of phytoplankton blooms. For this analysis we consider only the aggregate groups of diatoms and dinoflagellates. We aggregated the cell counts from all depths and taxonomic units within each group for each sample date. We then calculated the cumulative cell counts within each year beginning in January of each year. Each sum was divided by the annual total and plotted against the day of year. These cumulative curves indicated that the annual increase in diatom abundance began around d 110, reached 50% of its annual total between d 150 and 250 and had returned to background levels by about d 275. The spread in the median day was due largely to an early increase of diatoms in 1991 relative to the other years. The median day for the remaining 11 years ranged between about d 200 and 250. The cumulative curves for dinoflagellates indicated the annual increase began around d 150 or about 40 d later than for diatoms. The range in the median day for dinoflagellates was much narrower than for diatoms and ranged between about d 210 and 230 for most years. In 1998 the dinoflagellate cycle appeared to be early with 50% of the cells counted occurring by about d 180. A comparison of the cumulative curves for diatoms and dinoflagellates within each year shows that in some years (1988, 1994, 1995, 1998 and 1999) the dinoflagellate cycle preceded that of the diatoms whereas in other years (1989, 1991, 1992, 1993) the diatom bloom preceded the dinoflagellate bloom. This type of analysis was also conducted for an inshore station located within Lime Kiln Bay in the western Bay of Fundy in the heart of the local salmon aquaculture industry. In the future we hope to further refine and quality control our database and explore the temporal and spatial variability in the patterns more fully. For example, we hope to apply statistical methods to comparisons of the cumulative curves between years and locations, to detecting significant time patterns in inter-annual variability, for detecting patterns in community structure (including harmful algal blooms) and for exploring potential associations between the species abundance and environmental patterns.

THE CHALLENGES OF FORECASTING AND MANAGING TOXIC *PSEUDO-NITZSCHIA* BLOOMS ON THE U.S. WEST COAST

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The food web transfer of domoic acid to shellfish, crustaceans, seabirds, finfish and marine mammals has been recently documented on the U.S. West coast. Data collected during West Coast cruises in the years 1997-2000 indicate that often the highest toxin levels and greatest numbers of toxic cells are positioned in water masses associated with offshore eddies or in upwelling zones near coastal promontories. Such cruise data are essential in the characterization of offshore initiation sites that will lead to the effective placement of automated sensors, such as moored arrays. In addition, beach monitoring is a necessary component of regional species characterization, resulting in the development of specific molecular and biochemical tools needed to assist managers in each coastal area. Indeed, beach samples collected in 1998 indicated that a *Pseudo-nitzschia pseudodelicatissima* bloom was responsible for razor clam toxicity on the Washington coast, whereas toxin produced by *P. australis* resulted in sea lion mortalities in central California. The challenges faced on the West Coast due to HAB-related mammal mortalities, widespread closures of shellfish harvest, and human illness can only be met by sustained beach monitoring programs such as the Olympic Region Harmful Algal Bloom (ORHAB) project and dedicated research cruises. These onshore and offshore efforts will give us a comprehensive picture of the oceanography influencing the location and intensity of domoic-acid-producing HABs. Complete characterization of physical, biological and chemical conditions that favor harmful *Pseudo-nitzschia* blooms, only possible through large-scale, synergistic collaboration, is a prerequisite for forecasting of these events. A forecasting capability will substantially improve the management of valuable coastal resources and the protection of human health, both of which are affected by these toxins.

MONITORING OF FISH-KILLING ALGAE USING THE SANDWICH HYBRIDIZATION ASSAY

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The rapid detection and enumeration of fish-killing algae is crucial for the management of finfish farms. Cell detection methodology based on light microscopy can be tedious and time-consuming when large numbers of samples need to be processed. And for some species may require experienced personnel. To address these issues we have developed molecular methodology to provide rapid identification of harmful algal species in near real-time. Here we report on the use of sandwich hybridization technology to detect the raphidophytes *Heterosigma akashiwo*, *Fibrocapsa japonica* and *Chattonella* spp. Field testing of the sandwich hybridization assays (SHA) in New Zealand (*H. akashiwo* and *F. japonica*) and in Puget Sound, Washington, USA (*H. akashiwo*) has shown that the target organisms are detected below the level of concern. The SHA developed for *Chattonella spp.* has successfully detected laboratory cultures of *C. antiqua*, *C. marina* and *C. subsalsa*. In addition, *Chattonella* has been detected in Lugol's preserved field samples from Texas (confirmed by light microscopy). For the above species, no false positives have been returned using the SHA's against a wide panel of organisms, including natural samples replete with detritus and flocculent material. In addition, all the raphidophyte cultures examined from Australia, Germany, Japan, New Zealand, South Korea and the USA have been correctly identified using their respective SHA.

THE HARMFUL ALGAE MONITORING PROGRAM ON CANADA'S WEST COAST: WORKING WITH THE SALMON AQUACULTURE INDUSTRY

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The Harmful Algae Monitoring Program (HAMP) was initiated at the Pacific Biological Station (PBS) in June 1999, with the assistance of the salmon farming community. Funded by a consortium of companies, the program has expanded from three companies with 18 monitoring sites in 1999 to eight companies and 26 monitoring sites in 2001. Sites are situated around Vancouver Island at salmon farms or suspected seed areas for HAB's (Fig.1).

Fig 1. HAMP monitoring sites 1999 to 2001.

The program mandate was: to provide a HAB early warning system for the salmon farmers; to establish a central office for phytoplankton analysis and deposition of site environmental data; to standardise industry-wide sampling protocols; and to train and educate farm personnel in sampling and phytoplankton monitoring techniques.

A HAMP centre was established at PBS. Discrete water samples, collected from three depths weekly at the monitoring sites by farm personnel, are forwarded to the centre for algal species identification and quantification. Analysis of these samples concentrates on species known to be harmful to fish, but dominant species is also noted and quantified. Total biomass is approximated, on a scale of 1 to 5, plus percentage of biomass in five constituent groups: diatoms, dinoflagellates, raphidophytes, other flagellates, and microzooplankton. Collected environmental data from the sites are amassed at the centre, and water samples collected from selected sites are analysed for nutrient parameters (nitrate, phosphate, and silicate).

Weekly reports from March to November summarise the dominant and harmful phytoplankton species at the monitoring sites, and provide early warning of blooms developing on the coast.

This is facilitated by direct communication between representatives of the farm companies and the HAMP centre. Annual reports are produced at the end of each monitoring season. These reports discuss the dominant groups, biomass, and harmful species of phytoplankton at each site, and on the west coast in general. Any HAB's that occurred in the past year are noted. Environmental conditions and nutrient levels are also looked at in conjunction with the phytoplankton data.

Industry-wide protocols specifying sampling depths and methodology were established by HAMP. This prime objective of the program allows for uniformity in data collection and direct comparison of data from different areas and aquaculture companies. Phytoplankton workshops are held in different farm locations. These three-hour workshops train farm personnel in phytoplankton identification and enumeration, concentrating on known harmful species, using the resources normally available. In addition, algal physiology and morphology are discussed to provide a basic understanding of phytoplankton bloom dynamics. The *HAMP Harmful Plankton Handbook*, a reference manual for the fish-farmers that is updated annually, is distributed to workshop participants. Participation has increased in the first three years of the program: in 2001, 30 workshops, with 300 participants, were held on Vancouver Island and the north coast of British Columbia (Fig. 2).

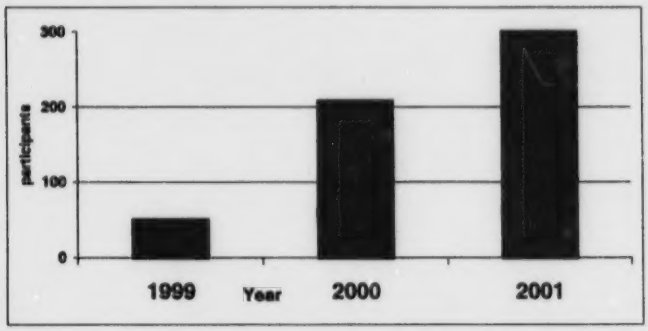

Fig. 2. HAMP workshop participants 1999-2001.

The database of yearly phytoplankton and environmental parameters will enable us to examine long term trends in HAB's at specific salmon farming sites. In the future this will provide a predictive capability for aquaculture site selection.

FIRST RECORD OF A *HETEROSIGMA AKASHIWO* BLOOM IN HOOD CANAL WASHINGTON, USA

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During September of 2000 the first recorded bloom of *Heterosigma akashiwo* (Raphidophyceae) in southern Hood Canal (Washington State, USA) occurred with cell numbers reaching 270 million cells L^{-1}. Fish kills from concentrations of *H. akashiwo* in marine waters have been reported from temperate regions worldwide (Taylor, 1990; Honjo, 1993). The economic losses incurred from the deaths of farmed salmon in the northeastern Pacific Ocean alone have reached into the millions of dollars (Black, 1990; Horner, 1999). Raphidophytes, including *H. akashiwo*, have been reported to produce brevetoxin-like compounds, potent neurotoxins (Khan *et al.*, 1997) and generation of superoxides may also play a role in fish deaths (Oda *et al.*, 1997). Mechanisms of toxicity induction in *H. akashiwo* blooms remain unclear with highly lethal and relatively benign blooms occurring in the same geographic area (e.g. Connell and Jacobs, 1999). Physical or biological conditions which may enhance toxicity are possibly very different from those conducive to bloom formation. Because of this alga's diel migration patterns and ability to swim, *H. akashiwo* competes well in stratified waters (Kohata and Watanabe, 1986).

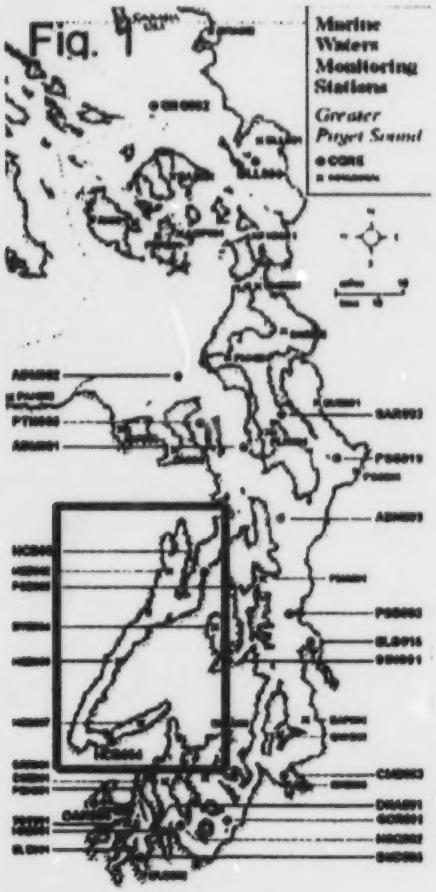

Fig. 1. Puget Sound Dept. of Ecology sampling sites. The Hood Canal is outlined

Hood Canal is a deep fjord-like appendage off western Puget Sound (Fig. 1). Historically, water samples from this area have occasionally contained low numbers of *H. akashiwo*. The waterbody is 50 km long, 2 km wide, an average of 100 m deep, with an entrance sill of 50 m depth. It has slow flushing times (~1 year). Persistent stratification is maintained from gradients in both salinity and temperature, with a strong pycnocline (δ sigma-t about 3-5) located at 5-10 meters depth. Strong water column stratification with low dissolved Oxygen levels are typical throughout the year but are more pronounced in the southern portion of Hood Canal and during the fall (Fig. 2).

On 9 September 2000 highly colored water was reported and dissolved oxygen concentrations were as low as 1.1 mg L^{-1} at 30m at in southern Hood Canal. Water discoloration covered up to 40% of Hood Canal as viewed by air, predominantly in the southern section. Recreational divers at Potlatch State park (September 10), near the southern end of Hood Canal, discovered dead starfish and crabs as well as oysters pulled out from the bottom (species not reported). A 300 m section of beach contained 3-5 dead fish (starry flounder *(Platichthys stellatus)*, gunnell species, greenling species) and shrimp species m^{-2}. Positive identification of *H. akashiwo* was made by Dr. R. Horner (University of Washington) based on morphological examination. The Washington State Department of Health (WSDOH) responded to reports of nausea and vomiting from two novice divers but the divers did not provide follow-up information and WSDOH concluded that there was not enough information or active cases to pursue an investigation. On September 14, 2000 *H. akashiwo* cell concentrations ranged from 2.7 x 10^8 cells L^{-1} in southern Hood Canal to 1 x 10^6 cells L^{-1} north near Lilliwaup. Higher cell concentrations were found on the west side of the Hood Canal than on the east side. By 17 September 2000 this bloom reached as far north as the Dabob river with substantially lower cell concentrations (3 x 10^5 cells L^{-1}).

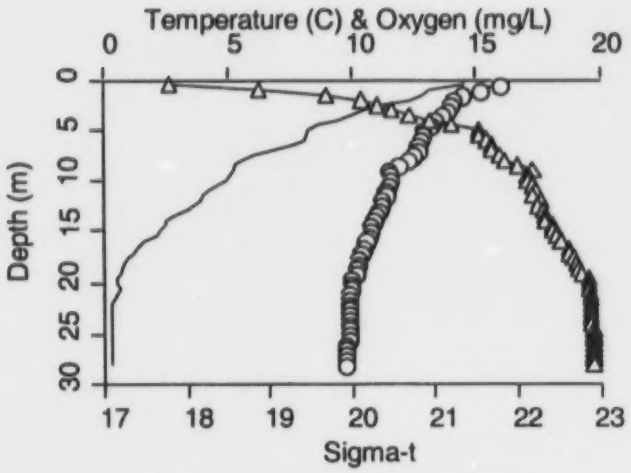

Fig. 2. Department of Ecology marine water monitoring data from 14 September 2000 showing strong stratification in southern Hood Canal near Sisters Point. Triangles represent values for density (sigma-t), circles for temperature and dotted line for oxygen.

In previous reports salmon have been observed to actively avoid *H. akashiwo* blooms (Hard 1999). In this case, Chinook (*Oncorhynchus tshawytscha)* salmon returning to the Hoodsport hatchery did not have any apparent adverse effects and did not avoid the bloom. These fish were observed to be holding outside of the hatchery in waters containing up to 1.4 x 10^8 cells L^{-1}.

The intensity of the bloom generated public uncertainty about potential health effects. As a result the Skokomish tribal subsistence fishery was voluntarily halted and the public beach at Potlatch State Park was closed for several hours. A recreational dive festival at Hoodsport proceeded as planned, however WSDOH staffers were on hand to monitor any public health effects. No additional human health complaints were recorded. Even though this was an intensive and massive *H. akashiwo* bloom, we believe that in this particular case fish and invertebrate mortalities were due to low dissolved oxygen rather than *H. akashiwo* toxicity. The public heath concerns resulting in closing of beaches, monitoring divers, and fisheries were the public greatest impact.

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NORTHCOAST PLANKTON IDENTIFICATION AND MONITORING PROGRAM

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The Northcoast Plankton Identification and Monitoring Program was designed to meet the following community needs of the Northcoast: (1) increased knowledge of marine plankton as they relate to mariculture; and (2) community participation and increased capacity in plankton monitoring. The purpose of the Northcoast Plankton Identification and Monitoring Program was to train local mariculture operators in the techniques of monitoring the location, density, species composition, and timing of phytoplankton and zooplankton populations in Northcoast waters in order to assist them in making informed decisions regarding harmful algal blooms, site locations, and timing of remote setting, grow-out, and harvesting operations. Additionally, the project collected plankton data for a period of one year at several sites in the Northcoast area, and attempted to correlate this data with PSP and domoic acid assays carried out simultaneously at the same sites.

A vertical haul was taken from 20 m to the surface using a 80 micron hand-held plankton net. The collected plankton were rinsed from the net into a labelled sample bottle and preserved using 2 mL of formalin for analysis in the laboratory. A one mL subsample was taken and examined microscopically using a Sedgewick-Rafter counting chamber. All organisms in the sample were enumerated, photographed, and identified to species where possible. Species densities integrated over the top 20 m depth were calculated from these data.

Ten study sites were selected in the Northcoast region: (1) Prince Rupert Harbour, (2) Humpback Bay Site 1, (3) Humpback Bay Site 2, (4) Holland Rock, (5) Rachel Island, (6) Metlakatla Bay, (7) Bernie Island, (8) Freeman Pass, (9) Hartley Bay, and (10) Rennell Sound. A year-long study was completed at three sites (sites 1, 2, and 3). PSP and domoic acid testing were performed at 6 sites (sites 2, 6, 7, 8, 9, and 10).

This study initiated a baseline for phytoplankton and zooplankton populations in the waters around Prince Rupert. To date, approximately 225 species of plankton have been identified in the study area. Although there was a great deal a variability among the study sites as a result of differences in oceanographic conditions, the following generalizations for the 2000 season could be made:

(1) A bloom of *Actinoptychus senarius* was seen between January and March at most sites (Fig. 1).
(2) Most diatom blooms occurred between May and August (Fig. 1).
(3) A small bloom of *Actinoptychus senarius* was seen in October at most sites (Fig. 1).
(4) The Shannon diversity index for diatoms ranged from 0 to 1.4. Some monospecific diatom blooms were observed at several stations.

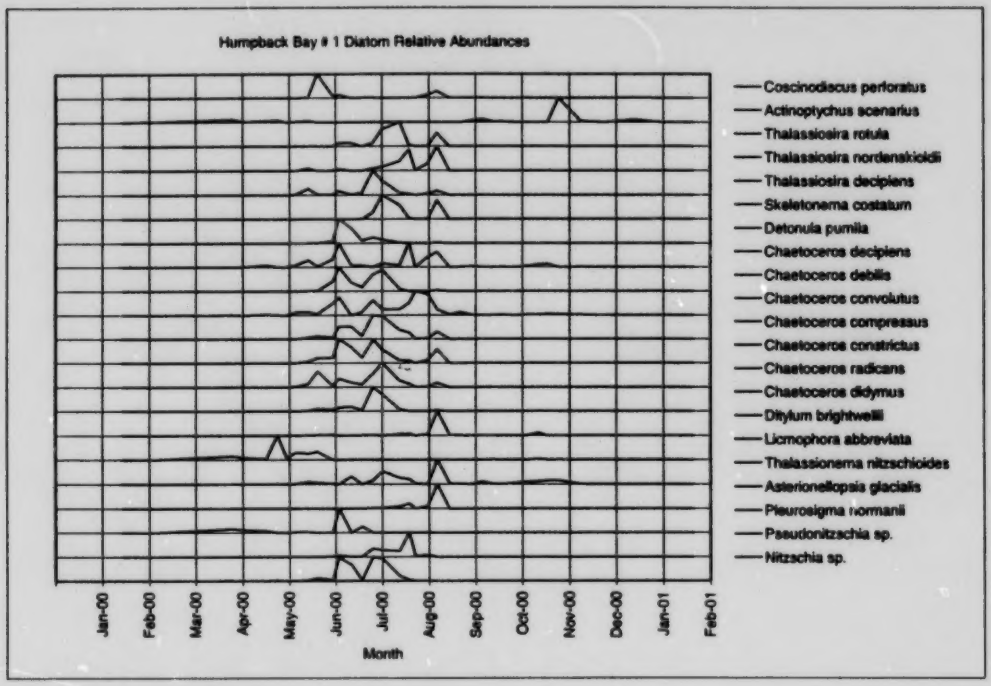

Fig. 1. Relative abundances of the dominant diatom species at Humpback Bay during the year 2000.

(5) The dinoflagellate season was initiated with *Gymnodinium lacustre* in April (Fig. 2).

(6) Most dinoflagellate species increased in abundance between June and November (Fig. 2).

(7) At most sites, the dinoflagellate season ended with the heterotrophic *Noctiluca scintillans* in November (Fig. 2).

(8) *Alexandrium tamarense* was present at significant densities from April through August (Fig. 2). Populations of *Alexandrium* spp. peaked 23.6 d, on average, before a response was observed in the mouse bioassay, and the threshold concentration of *Alexandrium* cells necessary to generate a measurable response in the mouse bioassay ranged between 3000 and 6000 cells/m^3 (Fig. 3). Both *Alexandrium tamarense* and *Alexandrium catenella* were present in the study area; however *Alexandrium tamarense* was the dominant species. Three sites in the study area had PSP results higher than 80 mouse units (shellfish closure limit) during the study: Bernie Island, Metlakatla Bay, and Humpback Bay. At Bernie Island, high toxicity occurred in May. At Metlakatla Bay and Humpback Bay, high toxicity occurred in August (Fig. 3).

(9) Both *Chaetoceros convolutus/concavicornis* and *Pseudo-nitzschia* spp. were present from June through August with maximum abundance during July (Fig. 1).

(10) All test results for domoic acid were negative.

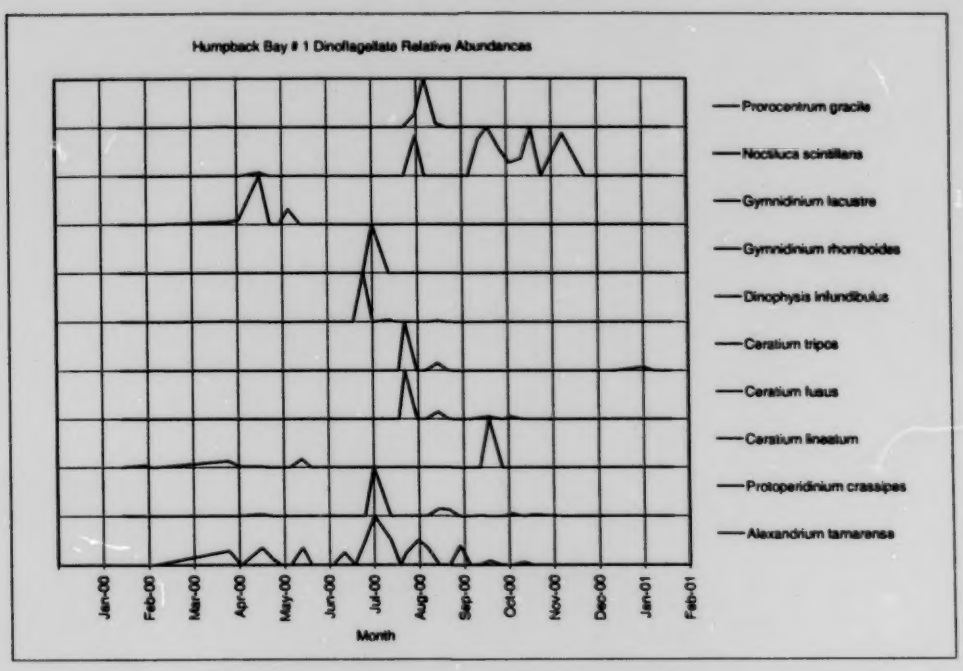

Fig. 2. Relative abundances of the dominant dinoflagellate species in Humpback Bay during the year 2000.

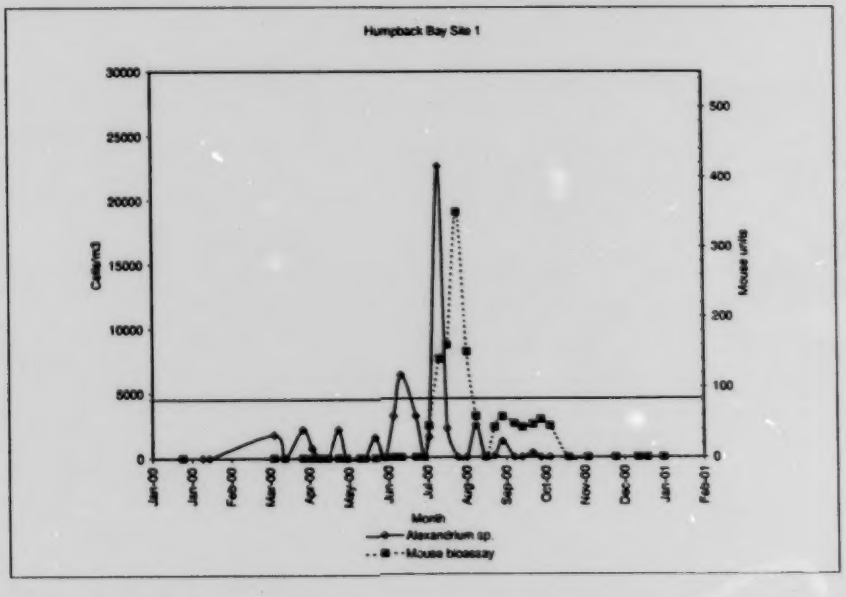

Fig. 3. *Alexandrium* spp. abundance and PSP mouse bioassay test results for Humpback Bay during the year 2000.

As a result of this project, twelve local people were trained in plankton sampling and preservation, and two were trained in plankton enumeration and identification. An interactive CD ROM for plankton identification was partially developed during this study, and will be used as a tool in further training offered by Ocean Ecology to people in the Northwest.

ORAL SESSION 4:

Emerging Assays for Marine Biotoxins

RAPID TOXIN TESTS: MIST ALERT™ FOR PSP AND ASP

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A novel rapid testing technology for marine biotoxins, based on lateral flow immunochromatography somewhat like that used in the home pregnancy test, has been developed by a Canadian company, Jellett Biotek Ltd. The tests, called MIST Alert™, are simple enough for field use and can be applied to the detection of marine biotoxins in shellfish or phytoplankton (Laycock *et al.*, 2000). One thousand one hundred and seventy nine shellfish extracts were tested in parallel using the mouse bioassay and MIST Alert™ for PSP in Alaska, Maine, British Columbia, Washington State, New Zealand and the UK. Further samples from the regulatory laboratory in California were tested in parallel in the Maine State laboratory. The MIST Alert™ for PSP detected 100% of the toxic (>80 μg/100g) sample extracts, and also detected the majority (94-95%) of extracts containing PSP toxin over 32 μg/100 g (Fig. 1).

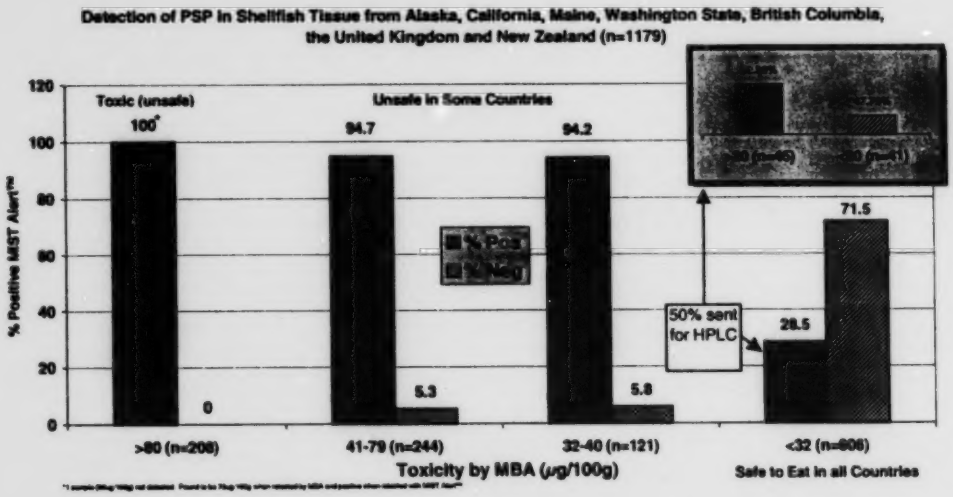

Fig. 1. Detection of PSP in shellfish tissue from Alaska, California, Maine, Washington State, British Columbia, the United Kingdom and New Zealand (n = 1179)

When toxicity was non-detectable in the mouse bioassay (<32 μg/100g), the MIST Alert™ test was positive 28.5% of the time (173 samples). This equates to a false positive rate over all trials of 15% when compared to the mouse bioassay. Fifty percent of the extracts that gave false positive results on the MIST Alert™ compared to the mouse bioassay were further analyzed by HPLC using the Oshima (1995) method. It was found that the MIST Alert™ detected toxicity that was present in 52.3% of the extracts analyzed by HPLC in the range of 20-40 μg/100g, below the level detectable by the mouse bioassay. Assuming a similar ratio for the remainder of the false positive (compared to the mouse bioassay) samples yet to be analyzed by HPLC, the MIST Alert™ for PSP appears to have a real false positive rate of only about 1-2%, when there is <20 μg/100g to no detectable PSP present. Because of the variation in toxin profiles found in naturally contaminated shellfish, the detection limit of the MIST Alert™ for PSP is about 40 μg/100g for the average profile, but may vary. Because of the variation in the profiles of toxin analogues, approximately 5% of the samples with mouse bioassay results between 32-80 μg/100g were negative over all the trials. However, there were differences seen in different geographic areas. For example, in the UK, where the toxin profiles were predominated by saxitoxin analogues for which the MIST Alert™ is most sensitive, all extracts with toxicity >40 μg/100g in the mouse bioassay were detected by MIST Alert™. Results of the parallel testing of the MIST Alert™ for PSP against the mouse bioassay are being published (Jellett *et al.*, 2001).

The newer MIST Alert™ for ASP has been 100% effective at detecting domoic acid in shellfish extracts from the UK, and is being more broadly validated. The detection limit of the MIST Alert™ for ASP is approximately 8-12 μg/g, or about half the regulatory limit. Both MIST Alert™ for PSP and MIST Alert™ for ASP can be performed using the same sample preparation, further simplifying testing. The application of MIST Alert™ to shellfish testing at the regulatory laboratory will reduce both costs and animal use. The applications of this technology to harvest management, process control and site selection in the shellfish industry have tremendous potential to enhance shellfish safety. The newest application under development for MIST Alert™, phytoplankton monitoring, could provide an early warning for shellfish farmers in the event of a toxic event (Silva *et al.*, 2001a, 2001b, 2001c). The latest information on test validation and new applications will be presented.

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A RAPID AND SENSITIVE BIOCHEMICAL ASSAY FOR PSP BIOACTIVES BASED ON MOUSE BRAIN SYNAPTONEUROSOMES.

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A membrane potential assay using mouse brain synaptoneurosomes was evaluated for the routine determination of PSP toxins in mussels and other species important to the shellfish industry of British Columbia. Membrane potential changes to synaptoneurosomes were monitored using the fluorescent probe rhodamine 6G. Standard saxitoxin was found to be a potent inhibitor of the membrane depolarizing effects of the sodium channel activator veratridine (IC_{50} = 4 nM). Likewise, shellfish extracts containing PSP toxins inhibited veratridine-induced depolarization. Neither saxitoxin or shellfish extracts had any discernible effect on the resting membrane potential of synaptoneurosomes. When synaptoneurosomal results for extracts of mussels (n = 120) and other shellfish (n = 29) were correlated with official mouse toxicity assay data there was very good agreement (r^2 = 0.84 and 0.86 respectively), indicating that the *in vitro* assay has utility for a variety of commercially relevant shellfish species. Comparisons also suggest that the mouse synaptoneurosome assay is of similar sensitivity to the official mouse toxicity assay. The synaptoneurosome fraction can be prepared quickly (approx. 40 min) and an individual assay takes under 7 min. Since 20 such assays can be performed using material from a single CD1 mouse brain, there is considerable opportunity for reducing the number of animals required in conventional PSP monitoring while retaining the same animal model. The stability of the synaptoneurosome fraction and nature of the fluorescence assay should permit high-throughput automated testing to be developed.

MARINE BIOTOXIN MONITORING IN NEW ZEALAND – TOWARDS REPLACING THE MOUSE BIOASSAY

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The shellfish monitoring programme in New Zealand was designed to monitor shellfish for the presence of neurotoxic shellfish poisoning (NSP), paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP) and diarrhetic shellfish poisoning (DSP) toxins, and for detection of gymnodimine in shellfish. With the more recent discovery of pectenotoxin, yessotoxin and esters of okadaic acid as potential contaminants of New Zealand shellfish this monitoring system has become increasingly cumbersome and expensive. Here we summarise work done towards replacing the mouse bioassay for detection of lipid-soluble toxins. We have developed instrumental (liquid chromatography-mass spectrometry, LCMS) methods for pectenotoxin, gymnodimine and yessotoxin, and effect-based assays for DSP and NSP toxins. DSP contamination is assessed using the protein phosphatase inhibition assay. NSP contamination can be monitored using the neuroblastoma assay. Using these combined methods all of the lipid-soluble toxins so far identified in New Zealand, plus azaspiracid, can now be detected in shellfish extracts without the use of animal testing. These methods have been established as research tools in the first instance. Work is continuing to integrate these methods and others into an efficient and effective monitoring system that can successively replace the mouse bioassay. Problems arising during the initial phases of this implementation are discussed.

PHYTOPLANKTON MONITORING USING A RAPID FIELD TEST: MIST ALERT™ FOR PARALYTIC SHELLFISH POISONS

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ABSTRACT

A recently developed lateral flow immunochromatographic (LFI) assay (MIST Alert™) was used to detect the presence of toxins associated with paralytic shellfish poisoning (PSP) in batch cultured plankton. Detection of PSP toxins was carried out on individual samples of between 1.0 – 4.0 X 10^4 cells of a highly toxic isolate of the marine dinoflagellate *Alexandrium tamarense* (clone AL18b) from eastern Canada harvested throughout the culture cycle. Positive assays of PSP toxins using the MIST Alert™ were consistently obtained with <100 cells per sample and no false negative responses were recorded. Moreover, no false positive responses or matrix interferences were found in any of the tested samples of this toxic species. Cellular toxin content and composition was also monitored in parallel by high-performance liquid chromatography with fluorescence detection (HPLC-FD). Toxic content and composition was similar to earlier findings by HPLC-FD analysis, and was quantitatively consistent with the test values of the MIST Alert™. On the basis of these encouraging results, it appears that this immunodiagnostic test could be used as an effective early indicator of toxic algae before they are consumed by shellfish at levels that would cause toxicity above the regulatory limit (80 μg saxitoxin equivalents per 100 g shellfish tissue). The rapid identification of PSP toxins using the MIST Alert™ in the water column and benthos could also provide an effective tool for broad scale mapping of toxic algal blooms and management of coastal zone aquaculture activities.

INTRODUCTION

Paralytic shellfish poisoning (PSP) is a potentially fatal form of food poisoning caused by the ingestion of shellfish, and more rarely finfish, that have fed on toxic blooms of marine dinoflagellates that produce potent neurotoxins (Hall *et al.*, 1990). In temperate waters incidents of PSP are most often linked to species of *Alexandrium*, whereas in tropical waters *Pyrodinium bahamense* var. *bahamense* is most often cited as the culprit species (Taylor, 1984; Steidinger, 1993). Chemical analytical techniques for the detection of PSP toxins in shellfish continue to improve (Oshima, 1995; Luckas, 2000), but there has been comparatively little consistent progress in the general application of novel methods for the rapid detection of these toxins in shellfish or phytoplankton.

Three of the most common methods used for monitoring harmful algal blooms of PSP toxin-producing species are: a) conventional microscopic identification of toxic species (Taylor, 1984); b) detection of toxicity by association with the presence of symptoms in the mouse bioassay

(AOAC, 1999); and c) analytical methods for the qualitative and quantitative determination of these toxins, e.g. using high performance liquid chromatography with fluorescence detection (HPLC-FD) (Oshima, 1995). Although over 50 countries maintain some level of harmful plankton monitoring, these conventional monitoring programs based upon microscopic analysis are labour intensive, time-consuming, and require extensive taxonomic training. Currently, the most commonly used and accepted method to monitor PSP toxins in shellfish is the intraperitoneal mouse bioassay (AOAC, 1999), which is routinely conducted by regulatory laboratories on shellfish tissues. Nevertheless, the mouse bioassay results can sometimes be difficult to interpret when death of mice occurs between 5-7 min since it can vary greatly before and after this period (Sullivan, 1993). Although the mouse bioassay has been used on rare occasions for the detection of PSP toxicity in plankton matrices (Cembella *et al.*, 1988), this method is not practical or sensitive enough for large-scale monitoring applications and it cannot be effectively used as an early warning of harmful algal blooms. Analytical techniques, such as HPLC-FD and more recently liquid chromatography coupled with mass spectrometry (LC-MS), provide accurate quantitative determination of toxin profiles, however this sophisticated capability is limited to major centres and it is therefore not practical to conduct a rapid assessment of toxins in shellfish and plankton matrices in the field.

A novel rapid test for detection of PSP toxins in shellfish, recently developed by Jellett Biotek Ltd. (Dartmouth, NS Canada), is based on lateral flow immunochromatography (LFI) (Laycock *et al.*, 2000). The MIST Alert™ has been designed as a screening test for potentially toxic shellfish and it can provide a rapid and qualitative indication of the presence/absence of PSP toxins. This test is simple enough for routine field and laboratory screening and it is highly effective in the detection of marine biotoxins in shellfish, as shown in collaborative studies in Canada, USA and the European Union (Jellett *et al.*, 2001a, 2001b). In this study, we investigated the development of a rapid and simple method for the extraction of PSP toxins from phytoplankton matrices that could be used with both the modified MIST Alert™ and analytical methods for PSP toxins, such as HPLC-FD. The toxin content of a highly toxic isolate of *Alexandrium tamarense* was monitored throughout a culture cycle to determine the feasibility of this extraction method and the potential application of the MIST Alert™ for the detection of PSP toxins in plankton matrices.

MATERIALS AND METHODS

Culture and growth determination of the toxic isolate

A triplicate culture of *Alexandrium tamarense* (isolate AL18b) from the St. Lawrence Estuary was grown in 2.8 L Fernbach flasks for 35 d at 16 ± 0.5 °C, and maintained at a photon flux density of 210 μmol m^{-2} s^{-1} on a light cycle of 14:10 (light:dark). Cultures were grown in L1 medium (Guillard and Hargraves, 1993) minus silicate and supplemented with the addition of 50 μM ammonium chloride (final concentration). All medium was prepared with 0.22 μm-filtered seawater (salinity 31 psu) from the NRC Aquaculture Research Station at Sandy Cove, Nova Scotia. Samples of 1mL were withdrawn from each flask at regular intervals throughout the culture cycle to determine the cell concentration by phase-contrast microscopy (250 X magnification). Triplicate cell counts using a 0.1 mL Palmer-Maloney cell counting chamber were conducted at all sampling times.

Protocol for harvesting toxic cells and extraction of PSP toxins

After determining cell concentration, a volume of culture that varied between 1-70 mL was harvested aseptically at d 0, 5, 7, 10, 12, 14, 17, 19, 21, 26, 28, and 31 from each flask. Harvested cells were placed into 15 or 50 mL Falcon tubes and immediately centrifuged at 3700 x *g* at 4°C for 20 min. The supernatant was aspirated away with a vacuum pipette leaving a small and tight phytoplankton pellet that was quickly frozen on dry ice for 5-10 min before storage at –20°C. Prior to extraction of PSP toxins, samples were thawed at 4°C and 500 μL of 0.1 M acetic acid was added to the phytoplankton pellet. The cell suspension was mixed well and either applied directly to the MIST Alert™ after dilution, or transferred into a 1 mL screw cap vial after filtering through 0.45 μm centrifuge spin-cartridges (Millipore Ultrafree-MC). Samples were kept at 4 °C prior to analysis. Only selected samples were sonicated (1 min, 50% pulse) prior to filtration since it was earlier found that detection of PSP toxins using MIST Alert™ did not require sonication after cells have been previously frozen.

Screening for PSP toxins by MIST Alert™

Direct detection of PSP toxins using the MIST Alert™ diagnostic test was carried out on both the whole cell suspension and filtrate within 30 min after toxin extraction. Samples were diluted 1 in 5 with Phytoplankton Buffer prior to application to the test strip. A total of 100 μL from each dilution was applied to each MIST Alert™ cassette and results were read after 20 min. Replicates of all positive samples were carried out until diluted samples no longer contained detectable amounts of PSP toxins as indicated by the response in the cassette strip to determine the minimum number of cells required for positive detection.

HPLC analysis

For HPLC-FD analysis, an aliquot of the cell filtrate (non sonicated) was transferred to a 200 μL glass autosampler vial. Analysis of the principal PSP toxins was performed by reverse-phase ion-pair chromatography followed by post-column oxidation with alkaline periodate to yield fluorescent derivatives according to modifications of the method of Oshima (1995) as detailed in Parkhill and Cembella (1999). Chromatographic profiles were determined by injections of 10 μL of the extracts and quantified with certified external standards (PSP-1C) provided by the Chemical Reference Materials Program (CRMP) of the Institute of Marine Biosciences, National Research Council of Canada.

RESULTS

Mean growth rate of *Alexandrium tamarense* (clone AL18b) cells was determined from the average of triplicate cell counts from each Fernbach flask throughout the culture cycle. Cell concentrations of *Alexandrium tamarense* increased from a typical initial slow growth from 213 cells mL^{-1} to reach a maximum of 35 x 10^4 cells mL^{-1} on d 31 at the beginning of the stationary phase. To determine toxin content and toxicity at each sampling point, 10 - 46 x 10^3 cells were removed from the culture each time (Table 1).

Table 1. *Alexandrium tamarense* (clone AL18b). Sampling frequency, total volume and number of cells harvested through a culture cycle.

Day	Vol. Harvested (mL)	Range of cells harvested x 10^4
0	70	1.49-2.13
5	30	1.03-1.18
7	30	1.98-2.82
10	12	2.17-2.83
12	10	2.67-3.53
14	6	2.26-3.90
17	4	2.77-3.73
19	2	2.17-3.10
21	2	2.33-3.32
26	1.5	2.82-4.60
28	1	1.90-3.25
31	1	2.07-3.53

Total concentration of toxins (% Molar) for each toxin analogue derivative remained relatively constant through the culture cycle of *Alexandrium tamarense* (clone AL18b). The least potent toxins, C2 toxins, are the most abundant with over 60% of the total concentration as indicated from the HPLC-FD analysis (Fig. 1). The concentration of two of three of the most potent carbamate toxins, neosaxitoxin (~ 20%) and gonyautoxins (~ <10%), also remained relatively unchanged throughout the growth cycle; although saxitoxin showed a relative increase up to < 10% between d 10 to 19 which coincides with the period of higher division rate.

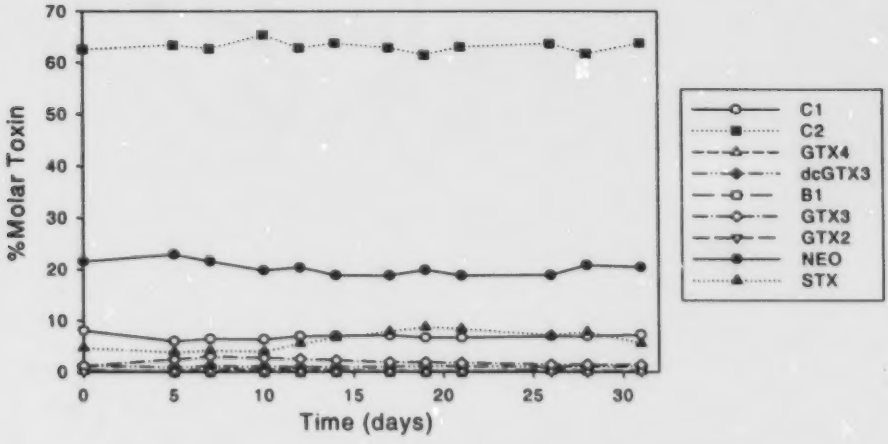

Fig. 1. *Alexandrium tamarense* (clone AL18b). Toxin composition (% Molar) for all major constituents C, NEO, STX, and GTX toxins for this highly toxic dinoflagellate grown under constant conditions of temperature, light and salinity.

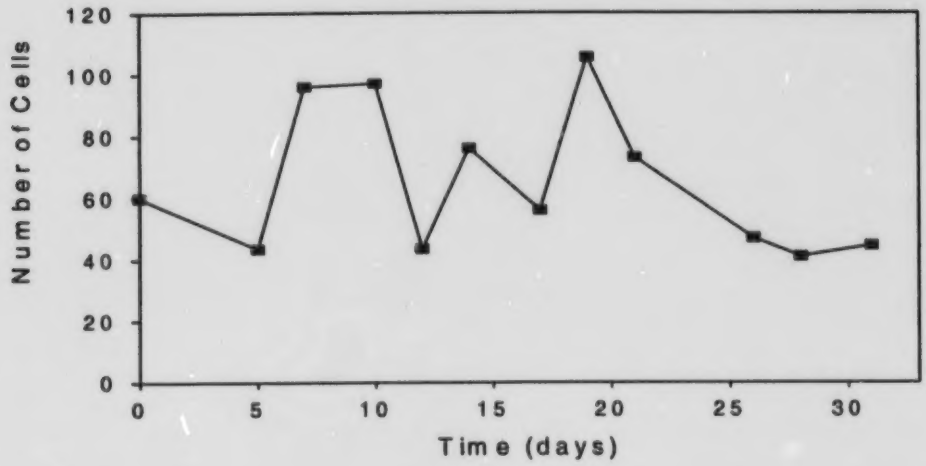

Fig 2. *Alexandrium tamarense* clone (AL18b). Minimum number of cells per 100 μL of sample required for positive identification of PSP toxins using the MIST Alert™ for PSP.

The range of number of *Alexandrium tamarense* (AL18b) cells required to positively identify PSP toxins using the MIST Alert™ test for PSP was well below 100 cells throughout the culture cycle (Fig. 2) and shows that cell toxicity was detected throughout the entire sampling period. Positive detection of PSP toxins was always found at the first dilution with phytoplankton buffer (1 in 5) of all extracts containing up to 2000 cells when tested by MIST Alert™.

The minimum range of detection of PSP toxins as depicted in Fig. 2, shows that the PSP toxin can be detected below 100 cells per test and as low as 20-40 cells per 100 μL (d 5). Above these cell numbers per test, PSP toxins are always positively identified.

DISCUSSION

Monitoring cell concentration and cell toxicity of *Alexandrium tamarense* (AL18b) in culture was feasible using a single and simple method of extraction and protocol for analysis of PSP toxins for MIST Alert™ and HPLC-FD analysis as indicated by confirmation of toxins by both methods. The relative higher toxin content and cell toxicity found at the boundary between late exponential and early stationary growth phase confirmed similar findings by Parkhill and Cembella (1999) for the same clone. The overall relative constancy in toxin production and cell toxicity found in this study resembles results reported earlier for other toxic dinoflagellates (Cembella *et al.*, 1987).

Positive assays of PSP toxins using the MIST Alert™ were consistently detected with <100 cells per sample during the culture cycle and no false negative responses were recorded. Moreover, no false positive responses or matrix interferences were found in any of the tested samples of this toxic species, either alone or when in assemblages with other toxic or non-toxic species. Although only data with the single 0.1 M acetic acid extraction method are presented here, 50%

methanol was also used with MIST Alert™ showing similar results. An improvement from previous methods is the fact that sonication did not appear to be necessary under the conditions used here as it made no difference in toxin profiles by HPLC-FD.

An early warning system that can detect the presence of PSP toxins in natural assemblages of phytoplankton could include the use of this MIST Alert™ test available with a few modifications in sample preparation. Potentially, this MIST Alert™ test could provide an effective tool for broad scale mapping of toxic algal blooms and management of coastal zone aquaculture activities.

ACKNOWLEDGEMENTS

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ORAL SESSION 5:

Public Health Issues and Phycotoxins Management

REPLACEMENT FOR BIOASSAYS IN SHELLFISH MONITORING; WHAT PROGRESS HAS BEEN MADE?

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In many countries use of mouse bioassays in shellfish toxin monitoring is unacceptable for ethical reasons. Pressures to introduce replacement techniques are applied from a number of sources including authorities tasked with enforcing animal regulations and the shellfish industry itself. Alternative methods have been discussed for a number of years yet few are implemented in countries conducting large scale monitoring of their shellfish industries. This presentation summarises the techniques available and the latest data on the evaluation of cell assays and the MIST Alert dipstick tests. It also describes development of other new approaches such as biosensors and evaluates their potential. Finally, it will discuss prospects for applying these techniques in regulatory scenarios.

TOXICOLOGICAL NEUROPATHOLOGY FROM DOMOIC ACID TO SPIROLIDES - THE HEALTH CANADA EXPERIENCE

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INTRODUCTION

The 1987 outbreak of shellfish related poisoning in Canada prompted greater collaboration between the National Research Council, Halifax, NS, Fisheries and Oceans Canada, the Canadian Food Inspection Agency and Health Canada in the study of naturally occurring seafood toxins. Domoic acid, a glutamate analogue and powerful excitotoxin, was identified as the toxin entering the food chain via blue mussels (*Mytilus edulis*) and subsequently consumed by humans (Thibault *et al.*, 1989; Teitelbaum *et al.*, 1990; Wright *et al.*, 1989). Domoic acid induced acute neurological, cardiovascular and autonomic symptoms with chronic memory impairment in some victims. Since 1987, HC has conducted several acute and sub-chronic studies in primates and rodents using different dosages and routes of administration (Iverson *et al.*, 1989; Iverson *et al.*, 1990; Tryphonas and Iverson, 1990; Tryphonas *et al.*, 1990a, 1990b; Truelove *et al.*, 1996). These data are the basis for the administrative guidelines of 20 μg/g in shellfish tissues. Kainic acid, a structurally similar excitotoxin found in seaweed, induces similar lesions to domoic acid. The introduction of immunohistochemical and transcriptional analysis has lead to further understanding of glutamate receptors (GluRs) as mediators of excitotoxicity (Gill *et al.*, 1998, 1999, 2000; Pulido *et al.*, 2000; Gill and Pulido, 2001). We have demonstrated the presence of GluRs in the heart and other peripheral organs (Gill *et al*, 1998, 1999, 2000; Pulido *et al.*, 2000; Gill and Pulido, 2001) suggesting a wider range of potential target sites for these neurotoxins. These observations were instrumental in the drive for the development of immunohistochemical and molecular methods as markers of neural injury. As a result, they are now being applied in the toxicological study of spirolides, a novel group of shellfish toxins (Richard *et al.*, 2001).

The Canadian Food Inspection Agency, the Department of Fisheries and Oceans, Health Canada and the seafood producers strive to ensure that Canada's seafood is safe. Each new aquaculture species or growing area brought into commercial production carries its own risks and monitoring challenges. In collaboration with responsible agencies, the Institute for Marine Biosciences regularly investigates anomalous results obtained in the routine monitoring program for shellfish safety. Although Canada has an abundance of clean water for shellfish production, bivalve molluscs are suspension-feeders that can accumulate naturally occurring toxins, which are unsafe

to animals and humans. The major sources of these toxins are certain species of microscopic algae that periodically occur in dense concentrations called "blooms".

Amnesic Shellfish Poisoning (ASP) and domoic acid

Amnesic shellfish poisoning (ASP) is the only poisoning syndrome caused by diatoms. The first recorded occurrence of ASP was in 1987 in Prince Edward Island, Canada, when approximately 100 people became ill and 3 others died after consuming contaminated mussels. The toxic agent was subsequently identified as domoic acid (DA) and the source was identified as the diatom *Pseudo-nitzschia multiseries* (formerly known as *Nitzschia pungens* f. *multiseries*) (Subba *et al.*, 1988). DA is an analogue of the endogenous excitatory amino acid (EAA) glutamate and is known to be a potent glutamate receptor agonist.

The symptoms of DA poisoning are gastrointestinal disturbances (vomiting and diarrhea), cardiovascular and respiratory symptoms including arrhythmia, hypotension and cardiovascular collapse. Neurological symptoms that are the most prominent include dizziness, disorientation, lethargy, seizures and death. Individuals of the 1987 outbreak who survived the initial toxic episode were left with permanent loss of short-term memory (Teitelbaum *et al.*, 1990; Zatorre, 1990). Several studies from our laboratory and others have demonstrated the acute and sub-acute effects of DA in mice, rats and monkeys (Tryphonas and Iverson, 1990; Tryphonas *et al.*, 1990a, 1990b; Truelove *et al.*, 1996). DA is similar in structure and function to another excitatory neurotoxin known as kainic acid (KA), which is found in the red macroalga *Digenea simplex*. DA and KA both appear to produce neurotoxic effects by activating the kainate (KA) and AMPA subtypes of glutamate receptors (GluRs) (Gill and Pulido, 2001). These receptors are ligand-gated ion channels that are activated by glutamic acid, mediating a fast excitatory synaptic transmission in the mammalian central nervous system. Persistent activation of KA receptors results in elevated levels of intracellular calcium (Ca^{2+}) through the co-operative interactions with NMDA and non-NMDA glutamate subtypes and voltage-dependent Ca^{2+} channels. Neurotoxicity caused by DA and KA appears to be similar, with results including high intracellular calcium levels and subsequent lesions in areas of the brain where glutaminergic pathways are heavily concentrated. This was particularly evident in the CA3 region of the hippocampus where neurons are sensitive to DA toxicity (Tryphonas and Iverson, 1990; Tryphonas *et al.*, 1990a, 1990b). Pyramidal neurons in the CA3 are 20 times more sensitive to DA than their counterparts in the CA1 in rat, mice and monkey. Activation of these receptors prompted seizures and the degeneration of neurons due to excitotoxicity. Long-term consequences of DA intoxication lead to disturbances in learning and memory processing (Carpenter, 1990; Teitelbaum *et al.*, 1990; Zatorre, 1990).

The histopathology of rodent hippocampus from animals treated with DA and KA is presented in Figs. 1a, 1b. The observations in the treated animals are: acute neuronal necrosis with vacuolization of the cytoplasm, cell drop out and edema of the neuropil. These lesions are characteristic of acute excitotoxicity of DA and KA. The only difference between these excitotoxins is their potency (DA >KA). Our experiments with KA showed that clinical manifestations occurred in older animals earlier than the younger animals. However the clinical, neurologic and histologic abnormalities were similar in both sets of animals regardless of age (Gill and Pulido, unpublished results). Although most experimental data emphasize the neuronal

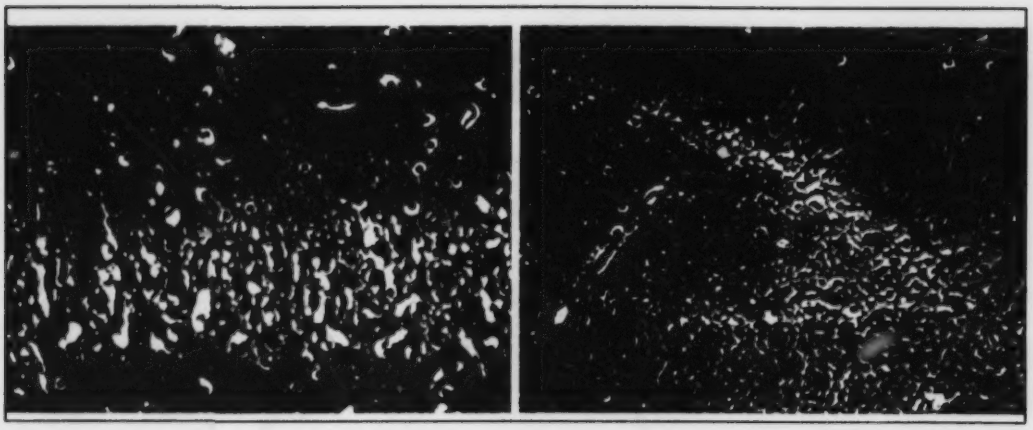

Fig. 1. Hippocampus of a rat treated with 4 mg/kg/ i.p. of domoic acid. Paraffin sections stained with H&E showing the CA 3 region (a) and the dentate gyrus (DG) (b). Acute neuronal necrosis, vacuolization of the cytoplasm, cell loss and edema of the neuropil are more prominent in the CA 3 than in the DG.

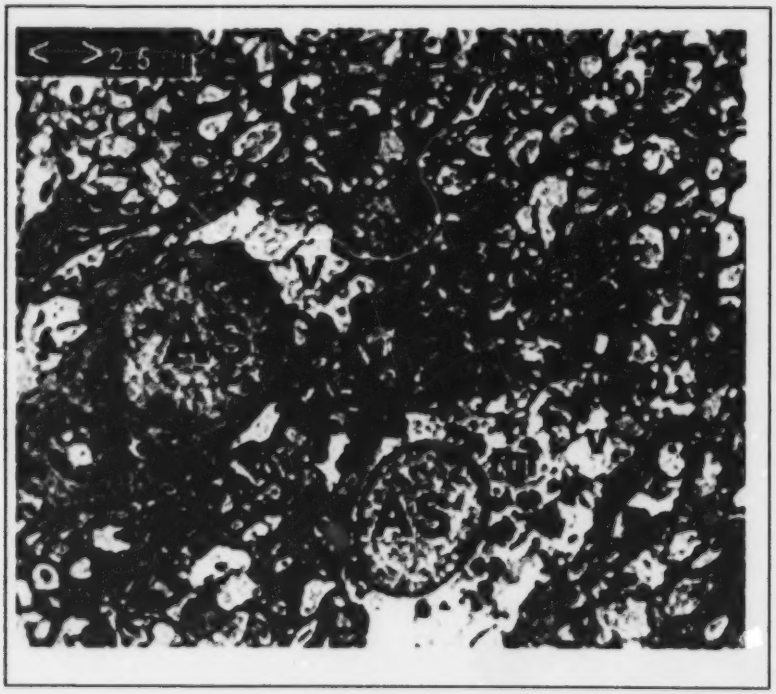

Fig. 2. Electron micrographs of the hippocampus from a rat treated with domoic acid with typical glial injury. Astrocytes (As) with vacuolated (v) cytoplasm contained small numbers of gliofilaments (gf) and remnants of damaged organelles. Injury of the astrocytes was more evident at the electron microscope level. These changes are consistent with recent publications denoting the importance of astrocytes as mediators of domoic acid toxicity and their protective role in mechanisms of neurotoxicity.

injury caused by these excitotoxins in the hippocampus, astrocytes also appear to be affected. Astrocytic changes were best visualized by electron microscopy (EM) of samples taken from the CA3 region of the hippocampus of rats treated with DA after fixation by perfusion with 2 % glutaraldehyde: 2 % paraformaldehyde (Fig. 2) (Pulido *et al.*, 1995). Ross *et al.* (2000) recently reported that DA induced decreased glutamate uptake in the rat astrocytes *in vitro*, thus supporting the view that astrocytes do contribute to excitotoxin injury (Ross *et al.*, 2000). Other regions of the central nervous system that were affected include the nucleus accumbens, the area postrema and the retina (Pulido *et al.*, 1995; Tryphonas *et al.*, 1990a, 1990b).

It has been established that DA and KA are known to mediate excitotoxicity through the over activation of the GluRs located on post-synaptic membranes (Gill and Pulido, 2001). Our observations support this view since immunochemical labelling shows a marked decrease in the stain intensity of antibodies for the GluRs subtypes KA and AMPA in the hippocampus of rats treated with KA. The microtubule associated protein (MAP2) antibody that preferentially binds to the microtubules of the dendritic tree shows a marked decrease in the immunostain in similar regions. These findings support the view of preferential dendrite injury by these excitotoxins. Animals that survived the acute toxic events and were alive for a longer period of time showed increased glial fibrillary acid protein (GFAP) immunolabelling. This increase in GFAP immunolabelling is only evident in animals that survived the acute lesions (Teitelbaum *et al.*, 1990).

The importance of the glutamate receptors as mediators of excitotoxicity has been reviewed recently (Gill *et al.*, 1998, 1999, 2000; Gill and Pulido, 2001). The demonstration that GluRs are present in heart and other tissues outside the CNS suggests that these tissues may be additional targets for excitatory compounds. The observations that the GluRs are present within the cardiac conducting system, intramural ganglia and cardiac nerve fibers could explain some of the clinical manifestations such as the arrhythmia described with DA intoxication in humans. In addition, Gulland (2000) showed histopathologic lesions in the heart of sea lions that died of DA intoxication in the coast of California in 1998. This report is the first to describe structural cardiac changes associated with DA poisoning. Hence individuals with pre-morbid cardiac conditions may be at higher risk of the toxic effects of these excitatory compounds. Therefore, the contribution of the GluRs in heart and the possibility of toxicity mediated through them needs to be determined (Gill and Pulido, 2001). Since the GluRs are omnipresent in different tissues and organs, we proposed a model of excitotoxicity as a common pathway for cell/tissue injury (Gill and Pulido, 2001). We are continuing our research based on this hypothesis because of the implications for food / therapeutic product safety and human health.

Spirolides - a novel group of shellfish toxins

In 1991, routine biotoxin monitoring of bivalve molluscs at aquaculture sites along the eastern shore of Canada, revealed the presence of a new type of shellfish toxin. Intra-peritoneal injections of the lipophilic extracts of shellfish caused a highly toxic and potent response in the mouse assay. The symptoms, which included rapid death preceded by neurological symptoms, were very different from those associated with other known lipophilic shellfish toxins. The symptoms of this 'fast-acting toxin' in mice after IP administration of contaminated shellfish extract were piloerection, abdominal muscle spasms, and hyperextension of the back and arching

of the tail to the point of touching the nose. Death was observed within 3-20 min. If a mouse survived 20 min, even while demonstrating symptoms, it usually recovered fully. Chemical studies showed that the toxicity was due to a family of novel seafood toxins, which were named spirolides (Hu *et al.*, 1995). Although there have been anecdotal consumer reports of vague symptoms (gastric distress and tachycardia) after shellfish consumption during periods when spirolides are present (May-July), spirolides have not been linked conclusively to any human intoxication.

Spirolides are macrocyclic imines existing in six forms: A, B, C, D, E and F, with E and F being non-toxic metabolites formed in the shellfish. They are similar in structure to other fast-acting toxins including pinnatoxins, gymnodimines (from *Gymnodium mikimotoi*) and prorocentrolides (from *Prorocentrum lima*). Using liquid chromatography-mass spectrometry (LC-MS) analysis, various spirolides of the A, B, C and D groups were detected in size-fractions of planktonic material from aquaculture sites. The dinoflagellate *A. ostenfeldii* was determined to be the causative organism (Cembella *et al.*, 2000). Spirolide-producing culture was obtained from Ship Harbour, Nova Scotia and was used to generate the biomass required for spirolides isolation, purification and initial toxicity trials using crude cell-free extracts (Richard *et al.*, 2001). The signs and toxic effects in mice administered intra-peritoneal (IP) and intra-gastric (IG) with aqueous extract of cultured *A. ostenfeldii* has been consistent with that of purified spirolides (Richard *et al.*, 2001). The toxicity of spirolides has been demonstrated in mice via both IP and IG, with estimated LD50 values of 40 μg/kg IP and 1 mg/kg IG. Initial pharmacological investigations with various compounds have shown that the time of death was accelerated when atropine and other acetyl cholinergic muscarinic receptor antagonists were used. On the other hand acetylcholine agonists offered protection hence extending the time of death. These investigations suggested that spirolides might act through muscarinic receptors, although other mechanisms have not yet been excluded (Richard *et al.*, 2001).

An acute pilot study was conducted to further elucidate the toxic effects of spirolides in the CNS and other organs/tissues. For this study, mice and rats were administered 75, 260 and 2000 μg/kg IP doses of highly purified 13-desmethyl-C spirolide. In both rats and mice, the animals died within 2 min of receiving the highest dose and within 8 min of receiving the lowest dose.

At the end of study animals were necropsied, followed by collecting various tissues for histopathology and transcriptional analysis. Since the clinical findings with spirolides suggested CNS effects, initial experiments were conducted on the CNS. For histological analysis, brains of mice and rats were fixed by immersion with 4% paraformaldehyde. The Mayer's hemotoxylin and eosin (H/E) and immunohistochemistry (IH) for selected neural markers such as glial fibrillary acid protein (GFAP), microtubule associated protein (MAP2), neurofilaments (NF160), silver and myelin stains were performed as previously described (Pulido *et al.*, 1995; Truelove *et al.*, 1996). For transcriptional analysis of mice, the brain was removed entirely and was snap frozen. For the rat, the brain was divided into the following two principal parts and snap frozen 1) cerebrum and 2) cerebellum and brain stem. Total RNA was extracted, pooled for each treatment group and used for semi-quantitative PCR. The biomarkers analyzed were the early injury marker (EIM)-Hsp72, c-fos and c-jun which are thought to be essential for converting stimuli into intracellular changes in neurons and the muscarinic acetylcholine receptors which are believed to be a possible mode of action of spirolides (Richard *et al.*, 2001).

For acute exposure in mice; histology data demonstrates that the hippocampus and brain stem are the main target regions. Astrocytic, endothelial and neuronal necrosis were observed particularly in mice receiving 260 and 2000 μg/kg of spirolides (Fig. 3). IH of neural markers showed diffused decrease intensity of MAP2 in the hippocampus of the treated animals as compared to controls. However, these findings need to be confirmed by other methods. Markers such as NF160 and myelin did not appear to be affected by the treatment. For the transcriptional analysis there were no alterations in any dose groups used of spirolides. For the CNS of the rat, there were upregulated transcriptional changes for all groups of biomarkers tested including EIM and the muscarinic acetylcholine receptors (M1, M4, M5). These changes were predominantly observed in the brain stem/cerebellum. However, in the rat there were no comparable histological changes at any of the equivalent doses used in mice.

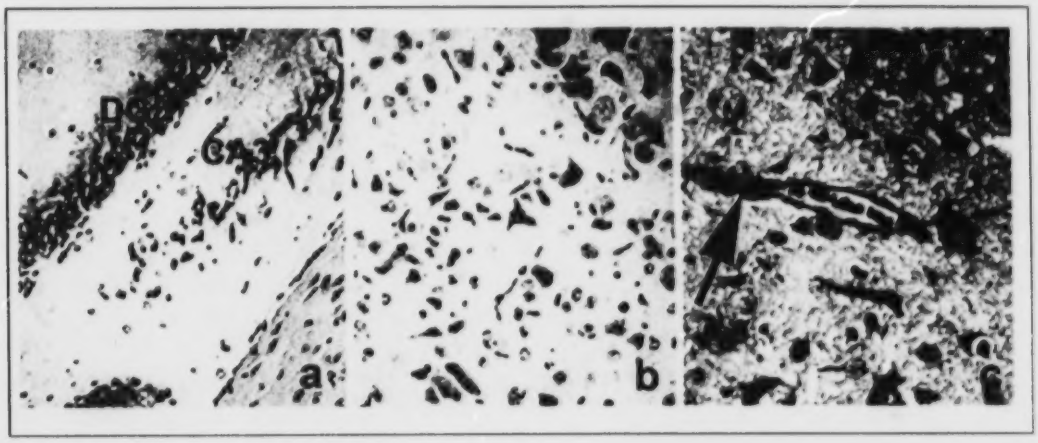

Fig. 3. Brain sections of a mouse treated with spirolide 2.0 mg/kg/ i.p. stained with H&E. The CA3 region and the dentate gyrus (DG) of the hippocampus (a) and brain stem (b and c) show acute diffuse cell injury when compared to controls. Neurons, glia and endothelial cells appear hyperchromatic with shrunken cytoplasm. The astrocytic lesion is best seen within the perivascular astrocytes (arrow).

Spirolides induce a rapid onset of toxic effects and lethality in both mice and rats at comparable dose ranges. Although the clinical symptoms were similar, histological and transcriptional data were different in the two sets of animals used. These results suggest that the effects of spirolides might be species dependent. It is possible that these differences could represent different phases of the toxic response to each of the treatment doses and species (Pentreath and Slamon, 2000). The other factors that could influence diagnosis of cell/tissue injury are detection time, survival time and conditions of exposure. Observations from this pilot study suggest that the brain is a target for this novel seafood toxin. In particular the brain stem appears to be a major affected region. This could explain the rapid death since the most important reflex centers of survival such as the cardiac, vasomotor and respiratory are all located in the brain stem. At the cellular level the astrocytes and the vascular endothelium appear to be target sites and could contribute to the toxic injury by altering astrocytic protective mechanisms and the blood brain barrier with consequent neuronal cell death (Pentreath and Slamon, 2000). The mode of action could

potentially involve the muscarinic receptors, since results from transcriptional analysis suggest that M1, M4 and M5 muscarinic receptors were altered. This is consistent the pharmacological findings of Richard *et al.* (2001).

The importance of seafood safety research, detection methods and monitoring was emphasized throughout this workshop. The data presented in this study support this continued proactive approach for food safety. This is particularly important since various seafoods are being enjoyed as a delicacy and nutritionally beneficial food. As demands and production of seafood increase, it is clear that more attention should be paid to seafood safety. Since our previous experience in the outbreak of domoic acid shellfish poisoning that occurred in 1987, it is clear that a program for monitoring seafood is necessary to ensure safe supplies. The understanding of the mechanisms of action for these seafood toxins is important to identify the appropriate preventive measures, the particular risk groups, and the proper treatment strategies.

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ADVANCES IN NEW ZEALAND MARINE BIOTOXIN MANAGEMENT

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Since New Zealand's first marine biotoxin event in 1992/1993, marine biotoxin management has made considerable advances. New Zealand now has a fully integrated programme involving early warning toxigenic phytoplankton monitoring, gene probe testing, mouse bioassay screen testing and confirmatory testing for ASP (HPLC), PSP (mouse bioassay), NSP (mouse bioassay), OA, DTX-1 (DSP ELISA Check kit or LCMS or PP2A), PTX's and YTX's (LCMS). The presentation will discuss how these advances have been progressed by researchers, regulators and the shellfish industry. Further developments are occurring with gene probe and LCMS technology. Problematic issues these advances raise for regulators, such as validation of LCMS methods, specificity of LCMS and the lack of standards will also be discussed.

ORAL SESSION 6:

Analysis, Effects, and Mechanisms for Production of Biotoxins

A YESSOTOXIN/DINOPHYSIS-TOXIN INCIDENT IN NEW ZEALAND CONFIRMED BY LC-MS/MS

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INTRODUCTION

In January 2001, positive mouse bioassays of lipid extracts (acetone and ether) and reported gastro-intestinal illness in shellfish consumers suggested the occurrence of a shellfish toxin contamination incident on the North-west coast of the South Island of New Zealand. This led to public health warnings against shellfish harvesting being issued. The first phytoplankton samples were collected from the area on 15 January. These revealed the presence of very high numbers of *Dinophysis acuta* (1.6×10^5 cells L^{-1}) and *Protoceratium reticulatum* (4.0×10^4 cells L^{-1}) within a visible "Red tide" extending for many tens of kilometres along the shore. This bloom lasted for a least 2 months and shellfish from some monitoring sites in the area remained positive (by mouse assay) until at least July 2001. Seawater and contaminated shellfish samples were analysed by liquid chromatography / tandem mass spectrometry (LC-MS/MS) and a variety of marine biotoxins were quickly identified.

SAMPLING AND ANALYTICAL METHODS

Plankton sampling revealed a highly stratified dinoflagellate community with high numbers of *P. reticulatum* in near surface waters and *D. acuta* in layers deeper in the water column. This made it possible to selectively collect relatively pure concentrates of the two species using a high volume centrifugal pump and a large volume plankton net (20 μm). Bulk quantities of contaminated Greenshell™ mussels (*Perna canaliculus*) were also collected.

LC-M/MS analysis of plankton concentrates and contaminated shellfish were carried out using a Waters 2790 LC system and 'Micromass Ultima', triple quadrapole mass spectrometer operating in multiple reaction monitoring mode (MRM) and using positive and negative electrospray ionisation. Chromatographic separation was performed using a Phenomonex Luna C_{18} column (150×2 mm) with 5 μ packing. A gradient from 13% acetonitrile to 77% acetonitrile was run between 2 and 10 min and held at 77% until 25 min. During gradient elution the phase was modified to give a constant composition of 2 mM ammonium hydroxide and 50 mM formic acid.

The MRM channels monitored were set up to run in windows that coincided with elution of the compound of interest from the chromatographic separation. The MS parameters are as follows: Electrospray positive, capillary 3.5kV; domoic acid 312.15>161.1 and 312.15>266.15, gymnodimine 508.4>392.3 and 508.4>490.3, PTX-2 876.7>823.5, PTX-1 892.7>839.5, PTX-2 seco acids 894.7>805.4, azaspiracid-1 842.6>672.6. Electro spray negative, capillary 3.0kV,

okadaic acid 803.5>255, DTX-1 817.5>255, 45 hydroxy yessotoxin, 1157.5>1077.5, yessotoxin 1141.5>1061.5.

RESULTS

Plankton concentrates containing very high cell numbers of *P. reticulatum* collected by plankton net from surface waters contained surprisingly low concentrations of yessotoxin (YTX) suggesting that a large proportion of the high concentration of YTX observed in whole water samples was dissolved in the seawater. There was no trace of 45OH-YTX in the plankton samples. Cultures of *P. reticulatum* isolated from this population have been shown to produce YTX at levels of 2-53 pg YTX cell^{-1}.

In the *D. acuta* cell concentrates low concentrations of okadaic acid (1.7-2.7 pg OA cell^{-1}) and relatively high and approximately equal concentrations of pectenotoxin 2 & 4 (PTX-1, PTX-4) were observed (22-82 pg cell^{-1}). Trace amounts of YTX (believed to be due to contamination from *P. reticulatum* cells) was present though there was no evidence of any DTX1, 45 OH-YTX, azaspiracid-1 or gymnodimine.

Analysis of the whole flesh of mussels collected from 3 sites along the affected coastline contained high levels of YTX, up to 326 μg YTX/100g. The shellfish also contained significant levels (about 22% of YTX) of 45 OH YTX. There was no evidence of DTX1 and only low levels (up to 13 μg /100g) of OA. Alkaline hydrolysis of extracts led to a five fold increase in OA indicating the predominance of esterified OA compounds ("DTX-3") within the diarrhetic shellfish toxin (DTX) complex. A similar magnitude of increase in OA was observed using the PP2A assay of hydrolysed extracts. High concentrations of PTX-2 seco acid (PTX-2SA) were identified (up to 1,132 μg/100g) at levels up to 20 times that of the parent compound PTX-2. Low levels of gymnodimine (~17 μg/100g) were also found in these shellfish. Selective analysis of the mussel hepatopancreas and the remainder of the tissues (gonads, gills, mantle, foot) showed an interesting variation in the distribution of the various toxins in these shellfish. With all the toxins except gymnodimine (YTX, 45OH-YTX, OA, PTX-1&4, PTX-1&4SA) >95% of the total toxin burden was located within the hepatopancreas. With gymnodimine, only approximately 5% of the toxin was found in the hepatopancreas with the majority sequestered within other tissues.

SUMMARY

This incident was the first time that LC-MS/MS analysis was used in near real time to confirm the nature of a marine biotoxin incident in New Zealand. It proved its efficacy to local public health authorities and shellfish quality regulators. These analyses revealed the real complexity of toxin profiles within shellfish contaminated during an incident of this nature. As LC-MS/MS is adopted as a routine monitoring tool it is likely that the identification of similarly complex toxin profiles will become common. Although the origin of okadaic acid, pectenotoxins and their various derivatives and yessotoxin could be definitely attributed to *Dinophysis acuta* and *Protoceratium reticulatum* respectively the origin of gymnodimine in these samples is unknown. It is possible that a bloom of a gymnodimine producing dinoflagellate (e.g. *Karenia selliformis*) occurred before the combined *D. acuta/P. reticulatum* bloom became established and the toxins produced by these species overlaid the gymnodimine already present in the mussels. In fact

mouse assays of acetone extracts had been showing positive results as early as October 2000 which may have been indicative of the original gymnodimine contamination event. This would be consistent with the majority of the gymnodimine being found in tissues outside the hepatopancreas.

As a result of the sampling carried out during this event, good quantities of plankton concentrate and contaminated shellfish have been collected. These will provide the raw material for the purification of the various toxins for use as analytical standards and reference materials.

ACKNOWLEDGMENTS

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NEW MARINE PHYCOTOXINS IN EASTERN CANADA

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Marine microorganisms such as phytoplankton produce a vast array of secondary metabolites, some of which are highly toxic to mammals. Since many of these same microalgae are food for shellfish and finfish, direct accumulation of such phycotoxins in edible tissues, or concentration through marine food webs, can result in human and animal poisonings. Phycotoxins are a serious threat to public health and have had significant economic impacts on aquaculture industries around the world. Prior to 1987, the only phycotoxins of concern to Canada were those responsible for paralytic shellfish poisoning (PSP). In 1987, a new toxin, domoic acid, caused a serious incident of amnesic shellfish poisoning (ASP) in Eastern Canada; in 1990, a few cases of diarrhetic shellfish poisoning (DSP) were caused by dinophysistoxin-1. Assorted live animal assays and liquid chromatography methods are now used to monitor shellfish for the presence of these toxins. One problem with this approach is that, since each method is specific, rigorous monitoring for all toxin classes requires the implementation of several methods and greatly increases the workload of regulatory agencies. New rapid assay methods appear to be very promising for the replacement of controversial animal assays as screening tools, but such methods usually require confirmation of positives by more sophisticated methods. Over the last decade, there have been dramatic improvements in instrumental analytical technologies for phycotoxin analysis. One approach that we have developed is the use of liquid chromatography-mass spectrometry (LC-MS), a very sensitive technique that can detect and identify many different toxins in one single analysis. In the year 2000, this technology was used to screen shellfish samples from Eastern Canada. The analyses revealed the widespread occurrence of two additional toxin classes: spirolides and pectenotoxins. The planktonic sources of these toxins have also been identified. The occurrence of these toxin classes in Canadian seafood presents new challenges to the aquaculture industry and the regulatory agencies.

DOMOIC ACID TOXICITY: NEUROTOXIC EFFECTS AND MECHANISMS OF SUSCEPTIBILITY

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Domoic acid is a tricarboxylic acid produced by certain species of the diatom genus *Pseudo-nitzschia*. It was identified as the causative agent of the amnesic shellfish poisoning in 1987 and since that time the toxic algae has been determined to have a worldwide distribution. This presentation will summarize the major points about domoic acid toxicity, including its toxicokinetics, adverse effects and mechanisms of susceptibility. Domoic acid binds competitively to kainic and AMPA subtype glutamate receptors and given to hippocampal pyramidal cells leads to rapid elevation of cytosolic free calcium. The elevation of calcium requires NMDA receptors, which are activated indirectly involving a mechanism of coincident detection. Domoic acid given at 1,2 and 4 mg/kg also causes a dose-dependent induction of the calcium-sensitive immediate response gene c-fos in brain tissue and induction of the product localizes to hippocampal formation, olfactory bulb structures and area postema/ nucleus solaritarius. Long term effects of domoic acid (2.0 mg/kg) in mice include decreased performance in working memory and (4.0 mg/kg) damage to soma, axons and terminals in hippocampus, lateral septum and olfactory bulb. However, the deficits in working memory can occur in the absence of apparent structural damage. The susceptibility to domoic acid has been examined using neonatal and repeated exposure models in laboratory mice. Neonates are forty times more sensitive than adults to domoic acid and the mechanism of increased susceptibility is poor clearance of the toxin. Repeated independent exposures of mice to domoic acid fails to show any additional symptomatic or behavioral toxicity. This information presented will be used to discuss practical approaches that can reduce the impact of domoic acid toxicity. These approaches include accurate communication of the hazards, identification of high-risk groups, and the prospects for biomonitoring.

IRON IS REQUIRED FOR DOMOIC ACID PRODUCTION BY *PSEUDO-NITZSCHIA MULTISERIES*

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The carboxylic acid residues of domoic acid (DA), the responsible agent for Amnesic Shellfish Poisoning, can bind trace metals such as iron. To investigate if the iron status of the cell affects DA production, replicate cultures of the diatom *Pseudo-nitzschia multiseries* were grown under trace-metal clean conditions in artificial f/2 medium containing 0, 0.12 μM and 11.7 μM added iron. All three iron treatments showed similar initial growth rates and stationary phase densities. In contrast, DCMU-enhanced fluorescence indicated that the cultures without added iron were iron-limited by d 15, as evidenced by Fv/Fm ratios of <0.2 as compared to the iron-replete cultures (11.7 μM Fe) of ca. 0.5. Cultures without added iron showed a marked drop in the cellular chlorophyll *a* content in stationary phase and never produced more than ca. 5 pg DA cell^{-1}. Iron-stressed cells also released significantly less iron into the medium at all times. In contrast, iron-replete cells contained 5-10 times more chlorophyll *a* per cell, and DA production increased during stationary phase to ca. 30 pg cell^{-1}. The intermediate level of added iron showed an intermediate response. Results of this latest experiment confirm that the lack of available iron strongly inhibits the ability of *P. multiseries* to produce DA in batch culture. The decrease in DA production under conditions of low iron may be explained by the requirement for iron in nitrate reduction and chlorophyll synthesis. An impairment of either of these processes would result in a lowered availability of nitrogen or photosynthetic energy, two factors that are required for DA biosynthesis. Although DA may chelate iron, our results suggest that DA is not produced as a way to enhance iron availability to *P. multiseries*. In fact, iron is now shown to be another essential factor for DA production. The physiological and ecological roles of DA still remain unknown.

ORAL SESSION 7:

Interactions of HAB Species with Other Organisms

ABUNDANCE, DISTRIBUTION, AND CHARACTERIZATION OF VIRUSES INFECTING *HETEROSIGMA AKASHIWO* FROM COASTAL SEDIMENTS OF BRITISH COLUMBIA

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Heterosigma akashiwo is a toxic bloom-forming alga that is responsible for extensive mortality of wild and farmed fish in British Columbia. The mechanisms that control the population dynamics of these blooms are not well understood. However, recent studies suggest viruses may play an important role. Since viruses are subject to removal and loss from the water column, they must be preserved in order to persist when their host is intermittently dormant. A possible reservoir for viruses is in the sediments, as viruses may attach to sinking particles or sink inside dying, infected host cells. We surveyed sediments from the Strait of Georgia and adjacent inlets to examine the abundance and distribution of lytic agents that infect *H. akashiwo*. Sediment samples were collected and processed to extract microbes smaller than 0.45 μm. Screening of the extracts against *H. akashiwo* cultures showed that 17 of the 20 locations sampled harboured lytic agents. Randomly selected samples were examined with TEM, and found to contain 50 nm virus-like-particles, suggesting most of these agents are likely viruses. The viruses originated from organic-rich and organic-poor sediments retrieved from depths ranging from 25 to 285 m. Viruses were detected within sediment cores as deep as 40 cm, suggesting the viable persistence of ~80 years. The highest abundance of viruses was 3210 viruses cm^{-3} from the sediment-water interface in Malaspina Inlet. Preliminary characterization of isolates suggests that these viruses do not belong to the established algal-virus family Phycodnaviridae. This indicates a previously unrecognized diversity among viruses that infect *H. akashiwo*, and therefore among algal viruses in general. Viruses in the sedimentary record indicate viral participation in past bloom dynamics, and provide a historical account of the distribution of *H. akashiwo* populations. It also suggests the potential role of viruses in controlling future populations of *H. akashiwo* if the viruses are resuspended into the water column.

THE DIVERSITY OF *HETEROSIGMA AKASHIWO* VIRUSES INDICATES THE COMPLEXITY OF VIRUS-HOST INTERACTIONS IN BLOOM DYNAMICS.

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Viruses may be important factors in bloom dynamics as regulators of bloom formation or termination. In order to study the impact of these mortality agents in nature, we must be able to identify and distinguish among them. Several viruses have been isolated from the Strait of Georgia, BC, which cause lysis of the toxic bloom-forming alga *Heterosigma akashiwo*. Two of these viruses have been characterized and they are unrelated based on their nucleic acid type, morphology, structural proteins, and the range of host strains that they infect. HaV 263 is a 25 nm, single-stranded RNA virus with a genome size of approximately 9100 nucleotides. The virus is sensitive to chloroform and contains at least 5 structural proteins. The infectivity of the virus was tested against fifteen strains of *H. akashiwo* from Japan, the Northwest Pacific, and the Northeast Atlantic. HaV 263 was able to cause lysis of four strains, but none from the Northeast Atlantic. HaNIV is a 50 nm, double-stranded DNA virus with a 37 000 base pair genome. This virus is also sensitive to chloroform, but contains only a single major capsid protein. HaNIV was able to cause lysis of six out of the fifteen host strains tested, but none from Japan. These two novel viruses do not resemble other known phytoplankton viruses and share few similarities with known virus families. This study shows the diversity of *H. akashiwo* viral pathogens. In addition, because different viruses infected different host strains, the complexity of virus-host interactions in the environment is demonstrated.

ALLELOPATHIC GROWTH INHIBITION OF *PROROCENTRUM MICANS* (EHRENBERG) BY *PROROCENTRUM LIMA* (EHRENBERG) DODGE IN LABORATORY CULTURES

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ABSTRACT

We investigated the possible growth-inhibition effect the toxic dinoflagellate *Prorocentrum lima* might have on other microalgae by employing the red tide dinoflagellate *Prorocentrum micans*. Both dinoflagellate species were grown in mixed cultures using complete and incomplete (lacking either in N or P) K-medium, at 24°C and 15°C. In all the study conditions, growth in *P. micans* was apparently suppressed in the presence of *P. lima*, and by the end of the fourth week cell number in the former species was between 2 and 10 times lower than in the latter. Therefore, we tentatively conclude that *P. lima* might be toxic to other microalgae.

INTRODUCTION

Several factors, acting individually or in synergy, are capable of influencing the development and blooming of microalgae. One of such influencing elements comes under the group name 'biological factors'. When we speak of biological factors, we also think of a possible ecological role of phycotoxins. That is to say, their contribution in the survival and blooming of species of microalgae that produces them. Actually the question that comes to mind is: do harmful microalgae produce toxins as a survival strategy, especially with respect to excluding potential competitors within the their community? Theories and tentative models of competition established to give a scientific basis to the "struggle for life" (Maestrini and Bonin, 1981), were stimulated first by observations made on animal species (Lotka, 1920, 1934; Volterra, 1926; Volterra and d'Ancona, 1935). Based on the outcomes, different theories have been advanced to describe respective behaviour of species inhabiting a niche and competing for the available resources there. Micro-organisms are not left out in these theories! And algal competition, for example, has been perceived as operating on the basis that the one species best able to obtain and assimilate any limiting nutrient should take the upper hand and outgrow the other competing species. However, the particular mechanism by which an individual inhibits the growth of another of the same or of different competing species is usually left vague, or just interpreted in terms of resources (Maestrini and Bonin, 1981); the rationale being that rates of growth decrease because the available food decreases with population increase. However, since it is claimed that oceanic phytoplankton is not nutrient limited, nutrient competition has been regarded as having little effect on the species competition and succession. Competition among microalgae is rather thought to be under such processes as selective grazing (Maestrini and Bonin, 1981). And it is on record that toxic microalgae are not usually grazed by zooplankton (e.g., Demaret *et al.*, 1995), and that where they are grazed upon, they cause serious casualties among the grazing population

(Demaret *et al.*, 1995; Ajuzie and Houvenaghel, 2000a, 2000b). Nevertheless, certain studies, for instance Shimizu (1987) and Mackenzie (1993), portrayed the toxin-producing ability of some microalgae as being rather whimsical or bizarre; the function of which is still mysterious. But, if we consider reports that have shown that herbivores do select what microalgae to graze and what not to (see Taylor, 1990), then we start to see some possible protective function of phycotoxins on cells that produce them. Works that report the avoidance of toxic cells by grazers abound. Such works include Ives (1985), Huntley *et al.* (1986) as well as Buskey and Hyatt (1995). The hypothesis is that a species that is capable of producing toxins that could kill or repel a grazer would certainly out-compete other species that are readily grazed upon. Thus, monospecific algal blooms have been interpreted in terms of grazer avoidance (e.g., Fiedler, 1982).

The production of extracellular substances by microalgae is well known (Hellebust, 1974; Nakamura *et al.* 1989). For instance, *Chlamydomonas* is known to produce large amounts of organic acids during its bloom, as evidenced from an increase in dissolved organic carbon in experimental media (Nakamura *et al.* 1989). Such extracellular substances produced by microalgae could also include toxins. *P. lima* is known to leak its toxins into the extracellular medium (Rausch de Traubenberg and Morlaix, 1995; Ajuzie and Houvenaghel, 2000a,b). The ecological importance of marine phytoplankton extracellular products is now recognized and such metabolites are considered to biologically condition natural waters in determining species dominance and succession (Smayda, 1974; Freeberg *et al.*, 1979).

This brings us to a supposed function of phycotoxins which is 'allelopathic inhibition' of other potentially competitive species (Taylor, 1990). In this regard, it is on record that *Gyrodinium* cf. *aureolum* exotoxins exert an exclusion inhibitory effect on diatoms (Arzul *et al.*, 1993). It was also the opinion of Freeberg *et al.* (1979) that the toxin produced by *Gymnodinium breve* allowed this species to dominate its other microalgal competitors and to form a massive bloom. While the growth of *G. breve* is inhibited by the external metabolites released by the cyanophyte *Gomphosphaeria aponina* (Eng-Wilmot *et al.*, 1977). And when *Prorocentrum micans* did not perform well in mixed culture with *Gymnodinium splendens*, Elbrächter (1976) concluded that the established inhibition of *P. micans* is not caused by lack of nutrients, but that *G. splendens* excretes one or more substances inhibiting growth of *P. micans*. Although Sugg and Van Dolah (1999) reported that there is no evidence for an allelopathic role of okadaic acid (OA) among ciguatera-associated dinoflagellates, Windust *et al.* (1996) believe the broad activity of the DSP toxins make them ideally suited to fulfil an allelopathic role in the biology of the producing organisms. Therefore, the present study was undertaken to investigate if *Prorocentrum lima* is capable of inhibiting the growth of *Prorocentrum micans* when cultured together. We think that there is paucity of information on interactions of this benthic alga with other microalgae. This is in agreement with Margalef *et al.* (1979) who opined that there is scanty work on biological interaction and succession among red tide organisms. And in the thinking of Windust *et al.* (1996), little is known about the effects of phycotoxins on marine organisms or their biological function. Anyway, some researchers (e.g., Aguilera *et al.*, 1993; Windust *et al.*, 1996, 1997, 2000) have focused on the effects of DSP toxins (OA and DTXs) on the microalgae community, but the effect which the DSP producing cells (e.g., *P. lima*) themselves have on other microalgae remains to be elucidated via laboratory experiments.

MATERIALS AND METHODS

Prorocentrum lima cells (strain PL2V) were obtained from Instituto Español de Oceanografía, Vigo, Galicia, Spain. And the *P. micans* cells were received from the Scandinavian Culture Centre for Algae and Protozoa, Botanical Institute, University of Copenhagen, Denmark. The microalgae were, thereafter, cultured and maintained in bacteria-free K-medium enriched seawater (Keller *et al.*, 1987) in our Brussels' laboratory. Seawater was obtained offshore from the English Channel, filtered under low vacuum on Whatman GF/C filters and autoclaved for 1 h before use. *P. lima* cells were grown at 24±1°C and at 60.19 μmol m^{-2} s^{-1} at a distance of ca. 10 cm from the light source; and *P. micans* at 15±1°C and at 23.95 μmol m^{-2} s^{-1} at a distance of ca. 20 cm from the light source. Both species were cultured on a 12:12-hr light and dark cycle. Increase in cell number (Figs. 1A, 1B), for the algal cells, was monitored via weekly microscope cell counts for one month, prior to the experiments.

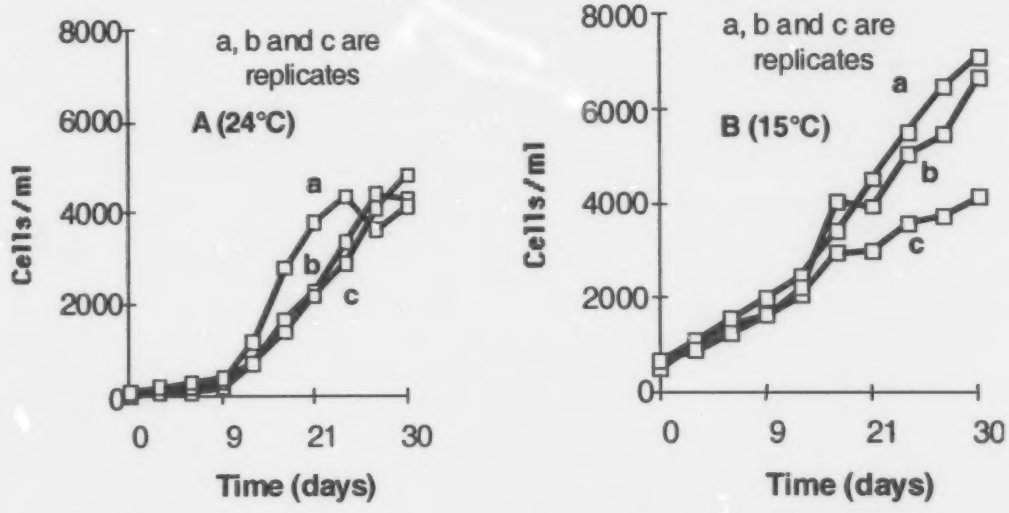

Fig. 1. Growth of *P. lima* (A) and *P. micans* (B) cultured separately in K medium for dinoflagellates during a 30-d culture period.

Investigations on growth inhibition of *P. micans* by *P. lima*

Both *P. lima* and *P. micans* were cultured together under different conditions of temperature and nutrient composition. Two culture incubators preset at 24±1°C and at 15±1°C, respectively, were employed during this study. Before the experiments were embarked upon, some *P. lima* cultures were acclimated for 1 wk in the 15±1°C incubator, while some *P. micans* cultures, on the hand, were acclimated at 24±1°C for the same period of time. Under these two different temperature progammes, the cells were cultured in K medium for dinoflagellates (Keller *et al.*, 1987). There were three treatments from the K medium. In one of the treatments the complete K-medium was employed, while in the other two a medium where either N or P was missing was employed. In the N-deficient medium, all nutrient elements in the K medium linked with nitrogen were

omitted, while in the P-deficient medium, all nutrient elements in the K medium linked with phosphorus were omitted.

The investigations were carried out in 58 mL test tubes containing 15 mL of the various media. Each treatment had a replicate. Cells were counted under the inverted microscope (in a 6-well Teflon-coated microscope slide; each well containing 50 μl of the culture of microalgae to be counted) on a weekly basis for four weeks. Before subsamples were taken for counting, the tubes were gently agitated to keep cells homogeneously suspended in the medium. Cells counted were converted to number per mL, summed up for the wells and for the replicates, and the averages plus standard deviations determined. The Fisher PLSD test (ANOVA) at 95 % significance level was used to compare growth rates at the two different temperature units for both species of microalgae. Additionally, a weekly ratio of cell numbers between *P. lima* and *P. micans* (i.e., *P. lima*/*P. micans*) was calculated, for the four weeks, in order to compute the degree of growth variations between the two species.

RESULTS

Nitrogen deficient K medium

In the nitrogen deficient medium and at both 24 and 15°C, growth of *P. micans* was lower than that of *P. lima* (Fig. 2A, 2B). Moreover, growth of *P. lima* was higher at 24°C than at 15°C. Thus, *P. micans*, at 24°C witnessed a steady decline after the first week (Fig. 2A). However, while *P. micans* showed a continuous increase in growth at 15°C, the rate at which it grew was not only lower than that of *P. lima*, but it also became slower and approached a plateau after the third week (Fig. 2B). The general observation, here, is that the growth of *P. micans* started to decline at the point where that of *P. lima* assumed a comparatively steep slope. Figs. 3A, 3B show that, in the absence of N, the difference in cell numbers between the two species increased as the weeks passed by, in favour of *P. lima*. At the beginning of the experiment, there was an approximate one-to-one cell ratio of both species in the two temperature programmes. But, by the fourth week, the *P. lima* cells out-numbered *P. micans* by, at least, a factor slightly greater than 2 (Table 1).

Table 1. Factor of difference in cell numbers between *P. lima* and *P. micans* (week 0 and 4)

	Factors of Difference* in					
	N deficient K medium		P deficient K medium		Complete K medium	
	24°C	15°C	24°C	15°C	24°C	15°C
Week						
0	0.80	0.91	0.80	1.02	0.77	0.89
4	9.95	2.34	5.14	2.87	3.82	1.88

* = ratio between cell numbers of *P. lima* and *P. micans*

Phosphorus-deficient K medium

Where phosphorus was lacking, *P. lima* also had higher cell numbers than *P. micans* both at 24 and 15°C (Fig. 2C, 2D). Generally, the growth of *P. micans* declined as that of *P. lima* increased at 24°C (Fig. 2C). However, at 15°C the growth of *P. micans* increased (but not at

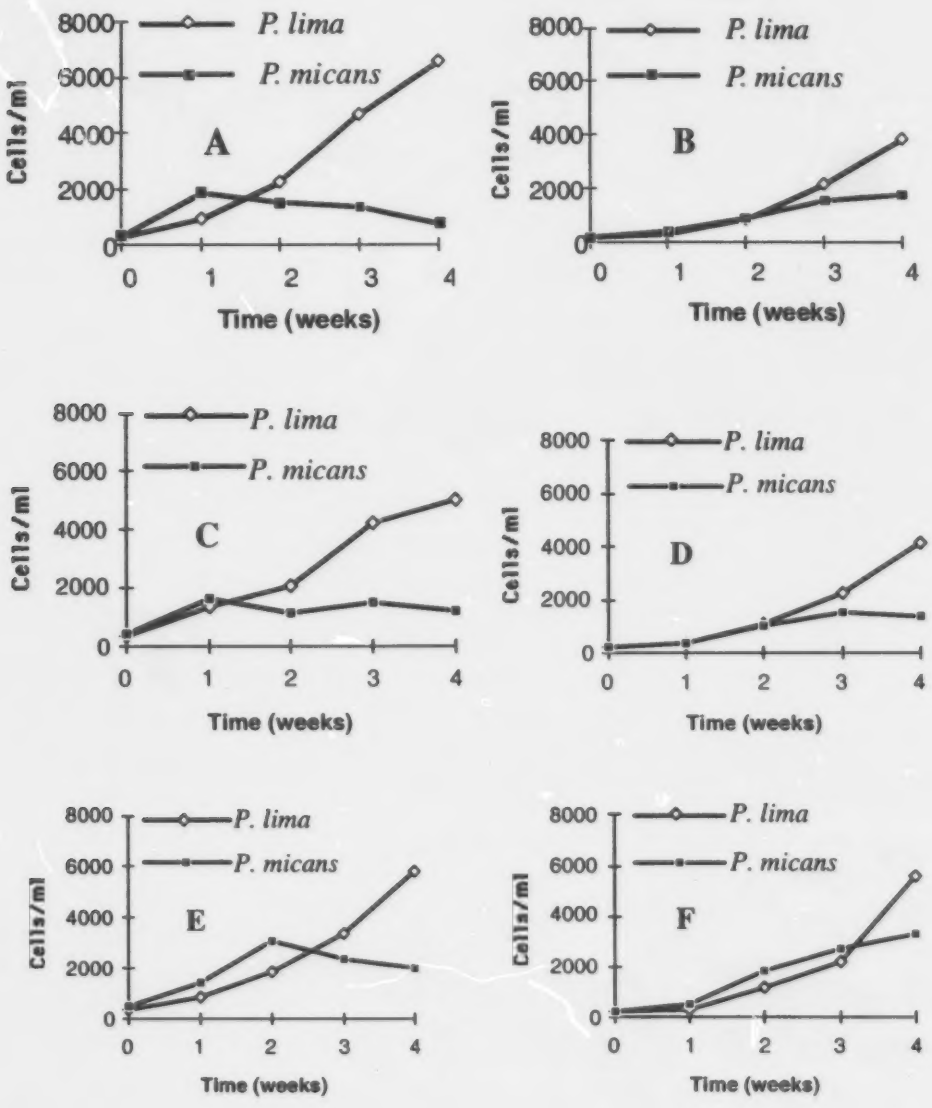

Fig. 2. Growth inhibition of *P. micans* by *P. lima* in laboratory cultures

A = N deficient K medium at 24°C
B = N deficient K medium at 15°C
C = P deficient K medium at 24°C
D = P deficient K medium at 15°C
E = Complete K medium at 24°C
F = Complete K medium at 15°C

the same rate as that of *P. lima*) until the third week when a decline in growth set in (Fig. 2D). As soon as the *P. lima* cells picked up higher division rates, the *P. micans* cells started to decline. Also, Figs. 3A, 3B show that, in the absence of P, the difference in cell numbers between the two species increased as the weeks passed by, with *P. lima* still on the lead. At the beginning of the experiment, there was also (approximately) a one-to-one cell ratio of both species in the two temperature programmes. But, by the fourth week, the *P. lima* cells out-numbered *P. micans* by, at least, a factor close to 3 (Table 1).

Complete K medium

Observations with the complete K medium were not different from those of the other two media, presented above; for *P. lima* still out-competed *P. micans* at both 24°C and 15°C (Figs. 2E, 2F). But while the growth of *P. micans* continued to rise at 24°C (even higher than that of *P. lima*) for the first two weeks, it fell sharply thereafter as *P. lima* cell started to divide at a faster rate (Fig. 2E). On the other hand, at 15°C the growth of *P. micans* was comparable to that of *P. lima* up to the third week, after which growth declined in the former following a higher rate of division (according to the relatively steeper slope between the third and fourth weeks) by the latter (Fig. 2F). Again, Figs. 3A, 3B show that the difference in cell numbers between the two species increased as the weeks passed by, again with *P. lima* dominating. At the beginning of the experiment, the cell ratio of both species in the two different temperature programmes was also one-to-one in the complete K-medium. But, by the fourth week, the *P. lima* cells out-numbered *P. micans* by a factor of ca. 4 at 24°C and a factor of ca. 2 at 15°C (Table 1).

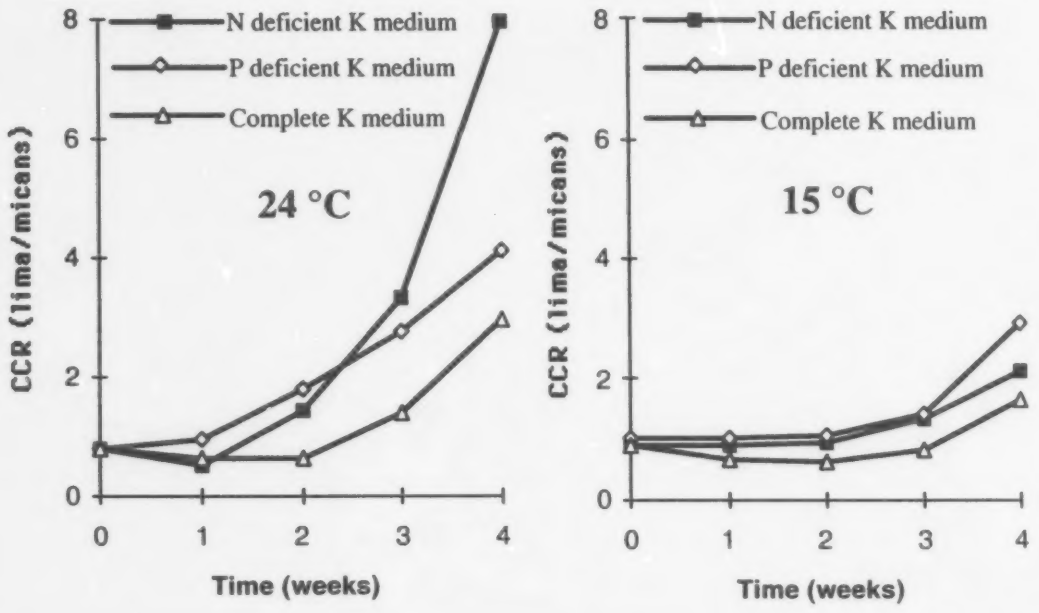

Fig. 3. Weekly cell concentration ratios (CCR) at 24 and 15°C (A and B).

A general observation was that while the two different temperature levels employed in this work seemed not to have influenced the growth rate of *P. micans* (mean cell number was not statistically significant at $p = 0.05$). The growth rate of *P. lima* (on the other hand) was faster at 24°C than at 15°C (Figs. 2 A-F). But the difference in mean cell number at the two temperature levels, for this species, was statistically significant ($p = 0.05$) only for the N-deficient medium. Another general observation was that the complete K medium apparently enhanced growth in *P. micans* more than did the N- and P-deficient media (though the difference was not statistically significant ($p = 0.05$). However, the enhanced growth (in *P. micans* in the complete K medium) declined by the second week at 24°C and by the third week at 15°C. By these times, the *P. lima* cells in the culture were growing at a relatively faster rate.

DISCUSSION

In all the experimental conditions, growth of *P. micans* was lower than that of *P. lima*. Low growth of *P. micans* was, particularly, more evident in the media that were deprived of either nitrogen or phosphorus. One would have been tempted to attribute the reason for these observations on the poor nutrient conditions of the N and P deficient media. But why was it that *P. lima* triumphed under the same conditions? And when the two species were cultured together in the complete K medium at 24 and 15°C, *P. lima* again triumphed, while *P. micans* populations declined by the fourth week of the experiments. But if we consider the fact that by the fourth week in K medium and when cultured separately, *P. micans* did not attain the stationary growth phase (Fig. 1B), we are left to assert that *P. lima* inhibited the growth of *P. micans* during these studies. If this was not the case, one would have expected growth in the two species, under the same treatment, to be the same since they belong to the same genus (congeneric) and, moreover, since they were acclimatized to the different temperature conditions prior to the experiments. Even at that, Figs. 1A, 1B show that the growth of both *P. lima* and *P. micans* can be supported by the K medium. So, *P. lima must* have been a problem source for *P. micans* in the mixed cultures.

P. micans, it should be remembered, is a planktonic species, while *P. lima* is primarily benthic. Therefore, with these observations in mind, one would have thought that *P. micans* would have had an edge over *P. lima* when cultured together since, as is stated by Steidinger and Tangen (1997), many planktonic species are bloom species. Faust (1993), in her description of *P. lima*, observed that this species rarely forms red tides. Nevertheless, Steidinger and Tangen (1997) also hinted that many benthic species are toxic and can reach high cell densities per square centimetre. So, the toxins of *P. lima* may be regarded as the mechanism through which this alga may exclude other competitors in its niche.

In nature, both of these species have worldwide distribution and are frequently encountered in neritic and estuarine environments (Steidinger and Tangen, 1997). In addition to these habitats, *P. micans* is also found in oceanic waters. Since the bottom layers of the aquatic environment are the store houses of nutrients, and since the neritic and estuarine waters are usually more exposed to eutrophication, it would not have been a surprise if *P. micans* out-grew *P. lima* in the media where either N or P was lacking; since being benthic and neritic, the latter species should have grown used to situations where nutrient supply is relatively high and not limited. But, as already seen, this was not the case. *P. lima*, rather, outgrew *P. micans* in all the treatments.

From the fore-going, we can only attribute the poor growth exhibited by *P. micans* (in the presence of *P. lima*) to growth inhibition of the former by the latter. The hypothesis is that *P. lima* leaked (see McLachlan *et al.*, 1994) its toxins into the external medium and that the toxins inhibited growth in *P. micans*. This hypothesis is put forward in view of the fact that crude extracts of the toxic *G. breve* tested for growth inhibition with 12 algal species resulted in the total arrest of growth for four diatoms and four dinoflagellate species (Freeberg *et al.*, 1979). Moreover, it has been established the culture medium of *P. lima* contains extracellular toxins (McLachlan *et al.*, 1994; Rausch de Traubenberg and Morlaix, 1995), and filtered culture medium of *P. lima* is toxic to brine shrimp (Ajuzie and Houvenaghel, 2000a,b).

A possible explanation for the growth inhibition effect of *P. micans* by *P. lima* is that the metabolic toxins produced by the former is (probably) capable of negatively affecting the growth of "unprotected" phytoplankters. Every cell produces secondary metabolites. Some of them have no clear function (and unless their formation is disadvantageous, evolution will not select against their synthesis), some may be repositories for waste or surplus products and some may be beneficial, for instance, by killing or discouraging competitors or predators (Flynn and Flynn, 1995). The metabolites of toxic microalgae have been shown to effect the latter impacts on predators (e.g., Fiedler, 1982; Ives, 1985; Huntley *et al.*, 1986; Buskey and Hyatt 1995; Pillet and Houvenaghel, 1995; Ajuzie and Houvenaghel, 2000a, 2000b).

Photosynthesis is vital for phytoplankton growth and division. So, when photosynthesis is adequately supported, the cell density increases. But if, for instance, the production of photosynthetic pigments is significantly affected by environmental or biological factors, microalgae productivity will decrease (e.g., Fábregas *et al.*, 1986, 1987; Jiménez and Niell, 1991). Therefore, another possible mechanism through which *P. lima* affected the growth and division rate of *P. micans* was, probably, through the inhibition of the synthesis of photosynthetic pigments in *P. lima*. Most of the *P. micans* cells were less pigmented towards the end of the study period. It should be borne in mind that toxins of *P. lima* are potent inhibitors of phosphatases 1 and 2A (PP1 and PP2A, respectively). Therefore, they have the potential to influence a broad range of cellular processes in eukaryotic cells, including plants (MacKintosh *et al.*, 1990).

During this study, it was observed that cell population of *P. micans* declined relatively sharply when the division rate of *P. lima* became apparently fast. Similarly, Kayser (1979) reported that competitive inhibition did not occur (among the dinoflagellates *Scripsiella faeroense*, *Prorocentrum micans* and *Gymnodinium splendens*) until maximum cell densities of the dominating forms had been reached. Thus, another hypothesis could be that as the *P. lima* cells divided, toxins escaped into the medium as the daughter cells were released, and that more toxins were released during higher rates of division.

Although P and N are essential nutrients for microalgae, vitamin B_{12} also plays an important role in the physiological development of microalgae. And the point that phytoplankton growth in the sea is ever limited by vitamin B_{12} deficiency (Droop, 1957, 1968) has been proved (see Menzel and Speath, 1962). Thus, it is not surprising that Keller *et al.* (1987) included this nutrient in their medium for culturing phytoplankton. Droop (1968) suggested that, "it is possible, for instance, that the concentration of binding factors or enzymes (permeases) at the cell surface

responsible for vitamin B_{12} uptake is controlled by the rate of cell metabolism, or it is possible that excretory products of one sort or another are responsible". Auto-inhibition of vitamin B_{12} uptake in phytoplankton has been reported. Also, the release (excretion) of vitamin B_{12} into the culture medium has been measured and found to be quite significant, though certainly too small alone to account for interferences with vitamin B_{12} uptake (Droop, 1968). Other excretory products of microalgae could play some significant role, in this regard. This leads us to reiterate that the toxins of *Prorocentrum lima* inhibited growth in *P. micans,* and that it did this by, probably, blocking the uptake of vitamin B_{12} (and, perhaps, the uptake of other nutrients in the growth medium) by the latter. *P. lima* actively leaks (excretes) its toxins to the external medium (see McLachlan *et al.*, 1994; Rausch de Traubenberg and Morlaix, 1995; Windust *et al.*, 1996; Ajuzie and Houvenaghel, 2000a, 2000b). Therefore, it is possible that the DSP toxins of this alga, in mixed cultures with *P. micans,* impacted the concentration of vitamin B_{12} binding factors on the cell surface of *P. micans*. Thus, by so-doing, they inhibited the uptake of vitamin B_{12} by *P. micans*. When supernatants of *Monochrysis* prevented the uptake of vitamin B_{12} by *Isochrysis galbana*, and thus inhibited the growth of the latter, Droop (1968) concluded that *Monochrysis* supernatants are sufficiently toxic to *I. galbana*.

Inhibitors have been classified as competitors and non-competitors. In the former category, inhibitors may combine with the substrate (nutrients), they may block the uptake sites or some part of the functional mechanism (Droop, 1968). In this regard, the DSP toxins have been viewed as potent blocking agents that, for example, inhibit the activity of protein phosphotases 1 and 2A. Due to their lipophilicity (as well as their unique and broad activity), the DSP toxins can readily fix onto the cell surface membranes of *P. micans* and, by operating from such vintage points, compete with the binding factors or enzymes (permeases) that usually occupy the cell surface and ensure the uptake of nutrients by *P. micans*. This may, thus, be one way by which *P. lima* makes itself obnoxious to other microalgae. Once *P. lima* had become dominant, the dominance would be effectively maintained by the inhibitor (the toxins); for subordinates would be prevented from taking up the nutrients and increasing in numbers. These subordinates will starve even in the midst of plenty, even though it would not matter that the dominant *P. lima* could no longer accumulate nutrients at an appreciable rate. This is very important since the specific growth rate of microalgae depends on the amount of nutrients in the cells and not on their medium concentration (e.g. see Droop, 1968).

Although Droop (1968) observed that all B_{12}-requiring phytoplankters he tested produced a B_{12}-binder and were inhibited by their own binder containing filtrates as well as filtrates from cultures of other species, the DSP toxins produced by *P. lima* are unlikely to auto-inhibition effects in this alga. Zhou and Fritz (1994) hinted that OA is likely to be produced by the chloroplasts of the cell and its compartmentalization in plastids may explain why producer cells are unaffected by its toxicity. Furthermore, Windust *et al.* (1996) observed that OA and DTX-1 inhibited the growth of non-DSP-producing microalgae, but not that of *P. lima.* They concluded that this differential activity of OA and DTX-1 suggests that the DSP toxins may play an allelopathic role. Apart from this, Aguilera *et al.* (1993) suggested that OA is a potent mitogene (induces mitosis in) of *P. lima*.

CONCLUSION

Allelopathic growth inhibition of *P. micans* by *P. lima* is suggested to have taken place in the various *in vitro* experiments investigated here. Therefore, since the DSP toxins (particularly, OA and DTX-1) have been proved to be potent microalgal inhibitors (Windust *et al.*, 1996), and since *P. lima* cells are capable of inhibiting growth in *P. micans*, we are tempted to believe that the hypothesis that DSP toxins are allelopathic has been tested in this work. Windust *et al.* (1996) remarked that "ample scope exists to examine the hypothesis that the DSP toxins are allelopathic using producing *Prorocentrum* species". Moreover, we are also tempted to suggest that OA and DTXs produced by *P. lima* may function in nature to inhibit the growth of other microalgae and that the inhibition would be of adaptive significance to this alga. Otherwise, how else can a poor swimmer like *P. lima* cope in a crowded niche with more active swimmers like *P. micans* that may quickly move away from such a crowded environment?

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BACTERIAL- MEDIATED INFLUENCES ON THE TOXICITY OF *PSEUDO-NITZSCHIA MULTISERIES*, A DOMOIC-ACID-PRODUCING PENNATE DIATOM

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Bacterial-phytoplankton interactions are increasingly becoming recognized as important factors in the physiology and dynamics of harmful algal blooms. Moreover, recent evidence suggests that bacteria can play a direct or indirect role in the production of biotoxins once solely attributed to microalgae. Evidence implicating bacteria as an autonomous source of biotoxins in PSP raises the question of autonomous bacterial toxigenesis of domoic acid (DA) in ASP. Here, we examine whether the previously observed bacterial enhancement of DA production in *Pseudo-nitzschia multiseries* cultures may be attributable to independent biotoxin production by the extracellular bacteria associated with this ASP-causing diatom. The growth and toxicity of nine non-axenic cultures of *P. multiseries* (clone CLN-1) was followed into stationary phase. During mid-stationary phase (d 14), three treatments were initiated. In Treatment 1, algal cells were removed using membrane filtration. For Treatment 2, flasks of *P. multiseries* were treated with antibiotics (penicillin:streptomycin 300:150 mg mL^{-1}) and tested for the absence of bacteria using two different sterility test media. The control, Treatment 3, continued to grow under non-axenic conditions with no *P. multiseries* cells removed and no antibiotics added. During the 24-d experiment, flasks were periodically sampled for cell number and for DA in the filtrate and "whole culture" (cells + medium), using FMOC-HPLC. Data were analysed using a two-way repeated measures ANOVA. Cell numbers remained statistically identical among treatments over the course of the experiment. Up to and including d 14, whole culture DA, and DA in the filtrate were not statistically different among treatments. After d 14, however, the non-axenic controls produced significantly more DA than did the axenic, antibiotic-treated cultures. This adds further support to previous findings that non-axenic cultures of *P. multiseries* produce significantly more DA than do axenic cultures. Following the removal of *P. multiseries* cells in Treatment 2, DA in the filtrate ceased to increase. Instead, DA levels continually declined; further experiments are examining this phenomenon. We conclude that after removing *P. multiseries* cells, the extracellular bacteria remaining in the filtrate were incapable of autonomous DA toxigenesis, even in the presence of *P. multiseries* exudates.

ORAL SESSION 8:

Spatial Distribution of Phycotoxins and Kinetics of Animal Toxicity

EXCRETION IS THE MAJOR PSP DEPURATION MECHANISM BY SHORT-NECKED CLAMS *TAPES PHILIPPINARUM*

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It is well known that PSP toxicity levels of bivalves differed from species to species, even those taken from neighbouring habitats. It is also known that some chemical and enzymatic toxin transformations of the toxins occur in shellfish. However, not much has been studied on the mechanism of toxin accumulation and depuration by bivalves and even it is not known whether degradation or excretion plays role in the depuration process of toxins. In this study, the mechanism of toxin depuration was determined on the short-necked clams *Tapes philippinarum*, which are one of the most popular species, having the third largest production in Japan. This species is also known to show rather low toxicity at *Alexandrium* bloom events. To obtain samples with uniform individual toxicity, the clams were fed with cultured *A. tamarense* every d for 8 d in 2 L tanks, then kept without feeding for 8 d more. Toxin contents in 6 clams were determined individually every 2 d by HPLC. Toxin contents in seawater, which was changed every d, was also analyzed by HPLC after concentration with an activated charcoal column, together with those in feces excreted by clams. Total toxin estimated to be lost from clams during non-feeding periods was almost explained by those detected in seawater samples. This result indicates that excretion is the major depuration mechanism of the short-neck clams and not much degradation is involved in the process.

SPECIES-SPECIFIC HEMOLYTIC ACTIVITY OF *HETEROCAPSA CIRCULARISQUAMA*, A NEWLY IDENTIFIED HARMFUL RED TIDE DINOFLAGELLATE

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ABSTRACT

Heterocapsa circularisquama, a recently identified novel species of dinoflagellate, has caused mass mortalities of bivalves in embayments of western Japan since 1988. Blooms of *H. circularisquama* can kill more than 12 bivalve species, but no harmful effects on wild fish populations, cultured fish, or on public health in general have been reported so far. Recently, the lethal effect of *H. circularisquama* on the pearl oyster (*Pinctada fucata*) was confirmed under laboratory conditions. In this study, we found that *H. circularisquama* produces species-specific hemolytic activity toward mammalian erythrocytes. Interestingly, sensitive erythrocytes are from rabbit, mouse, and guinea pig, all of which have K^+ as the main intracellular cation and its concentration is extremely higher than Na^+. The resistant erythrocytes are from human, cattle, and sheep, which have Na^+ as their main inside cation, except for humans. The species-specific hemolytic activity of *H. circularisquama* may partly reflect its strict species-specific toxic behavior. *H. triquetra*, which is morphologically similar to *H. circularisquama* but not toxic to bivalves, showed no hemolytic activity toward rabbit erythrocytes. Culture supernatants of *H. circularisquama* showed a weak but significant hemolytic activity. Hemolytic activity was found in ethanol extracts of *H. circularisquama* cells, suggesting that the hemolytic agents can be extracted into ethanol. Both an intact flagellate cell suspension and the ethanol extract caused morphological changes and eventual collapse of unfertilized eggs of the Pacific oyster. Furthermore, the ethanol extract was lethal to the microzooplankton rotifer *Brachionus plicatilis*, which is highly sensitive to *H. circularisquama*. Our results suggest that a hemolytic toxin produced by *H. circularisquama* may be one of the causative agents responsible for the shellfish toxicity.

INTRODUCTION

Heterocapsa circularisquama has caused mass mortalities of bivalves in embayments of western Japan since 1988 (Horiguchi, 1995; Matsuyama *et al.*, 1996). However, no harmful effects on wild fish populations, cultured fish, other marine living organisms or on public health in general have been reported so far (Yamamoto and Tanaka, 1990; Matsuyama *et al.*, 1992). Although detailed toxic mechanism of this flagellate is still unclear, Matsuyama *et al.* (1997) have speculated that unstable toxic substances located on the surface of the *H. circularisquama* cell may be responsible for the toxicity to bivalves. Furthermore, Matsuyama (1999) has reported

that an influx of Ca^{2+} was induced in trochophore larva of short necked clams (*Ruditapes philippinarum*) after exposure to *H. circularisquama*. Some marine toxins, such as palytoxin (Habermann, 1989) and maitotoxin (Igarashi *et al.*, 1999) are known to induce Ca^{2+} influx into mammalian erythrocytes and eventually caused hemolysis. These findings prompted us to examine if *H. circularisquama* produces hemolytic activity. In this study, we demonstrated that *H. circularisquama* caused hemolysis of rabbit erythrocytes, whereas *H. triquetra*, which is generally known as a non-toxic dinoflagellate, showed no such activity. The hemolytic activity was found in the ethanol extract of *H. circularisquama* cells, and the extract exhibited toxic effects on unfertilized eggs of Pacific oyster and microzooplankton rotifers (*Brachionus plicatilis*), comparable to results obtained with *H. circularisquama* live cell suspensions.

MATERIALS AND METHODS

Organisms and culture conditions

Clonal cultures of toxic and less toxic strains of *H. circularisquama* were originally isolated in Ago Bay and Imari Bay, Japan, respectively. *H. triquetra* was isolated in Hiroshima Bay, Japan. All these clonal species were cultured at 26°C in sterilized Erd-Schreiber modified (ESM) medium (pH 8.2) on a 12:12 light:dark cycle of 30 μmol photons m^{-2} s^{-1} (Oda *et al.*, 1992). Each flagellate culture was sampled at late exponential growth phase to test reactivity toward erythrocytes. The cell density of each culture was estimated using a hemocytometer. Adults of Pacific oysters (*Crassostrea gigas*) were collected from Hiroshima Bay. Eggs were sampled from a female (6.2 g). To minimize bacterial growth, 20 μg mL^{-1} of streptomycin was added to the media and the water temperature kept below 15°C until the assay. Resting eggs of the rotifer *Brachionus plicatilis* were kindly provided by Dr. A. Hagiwara (Faculty of Fisheries, Nagasaki University). The hatching of eggs was carried out under the appropriate conditions as described previously (Balompapueng *et al.*, 1997). Hatched neonates were pipetted out and used for flagellate exposure experiments.

Hemolysis assay

Rabbit, guinea-pig, sheep, and cattle blood were obtained from Nippon Bio-Test Laboratories (Tokyo) and used within 7 d. Freshly collected blood from humans and mice were immediately mixed with 0.1 volume of 10% sodium citrate to prevent blood coagulation, centrifuged, and the serum and buffy coat were removed. Erythrocytes of each species were washed three times with phosphate-buffered saline (PBS) and brought to a final concentration (v/v) of 4% in Erd-Schreiber modified (ESM) medium. Triplicate 50 μL aliquots of indicated concentrations of flagellate cell suspensions or the ethanol extract in ESM medium were added to the wells of round-bottom 96-well microplates (Falcon). To each well, the same volume of 4% (v/v) suspension of erythrocytes in ESM medium was added and the plate was gently shaken. After incubation for 5 h at 26°C under illumination from a fluorescent lamp (30 μmol photons m^{-2} s^{-1}), the plate was centrifuged at 900 x *g* for 10 min. Fifty μL of each supernatant was transferred to a flat-bottom microplate and the released hemoglobin absorbance at 560 nm was measured with a microplate reader (TOSOH, MPR-A4i). Zero hemolysis (blank) and 100% hemolysis controls were determined using erythrocytes suspended in ESM medium alone and 1% Triton X-100, respectively.

Preparation of culture supernatant and ultrasonic ruptured cell suspension of *H. circularisquama*

The culture supernatant was obtained from 10 mL of a late exponential culture (10^5 cells mL^{-1}) of *H. circularisquama* by centrifugation at 1000 x *g* for 10 min at 4°C. The ruptured cell suspension was obtained by sonicating 10 mL of a late exponential culture (10^5 cells mL^{-1}) for 60 s at 20°C in bath-type sonicator. Microscopic observation confirmed that all cells were lysed by this treatment.

Preparation of the ethanol extract

Harvested cells from 100 mL of the culture (10^5 cells mL^{-1}) by centrifugation (1000 x *g* for 10 min at 4°C) was resuspended in 100 μL of 100% ethanol and vigorously agitated by sonication. The lysate was centrifuged at 13,000 x *g* for 10 min at 4°C and the supernatant was withdrawn and stored at -30°C until use as the extract.

Effects of *H. circularisquama* and its ethanol extract on unfertilized eggs of Pacific oyster and rotifers (*Brachionus plicatilis*)

To examine the effect of *H. circularisquama* on unfertilized eggs of Pacific oysters, 1 mL of flagellate cell suspension (10^5 cells mL^{-1}) and 1 mL of an oyster egg suspension in ESM medium (10^4eggs mL^{-1}) were added to a well of 6-well plates. To examine the effects of the *H. circularisquama* ethanol extract on the oyster eggs, the ethanol extract was diluted with ESM medium to make a 2% solution and mixed with an equal volume of the oyster egg suspension in a well of 6-well plate. The plates from both treatments were incubated at 26°C under illumination from a fluorescent lamp (30 μmol photons m^{-2} s^{-1}). At 1 h incubation intervals, 200 eggs in each well were scored using a stereomicroscope and the number of morphologically intact eggs was determined.

To study the effects of *H. circularisquama* or the ethanol extract on survival of the rotifer *Brachionus plicatilis*, varying concentrations of the flagellate cell suspensions or the ethanol extract were prepared with ESM medium. One mL of cell suspension or the ethanol extract was placed in each well of 24-well plates and then 10 individuals of *B. plicatilis* were inoculated into each well. The plates were incubated at 26°C under illumination from a fluorescent lamp (30 μmol photons m^{-2} s^{-1}). A stereomicroscope was used to count viable individuals swimming in each well after a 5 h incubation.

RESULTS

Hemolytic activity of *H. circularisquama* toward mammalian erythrocytes

As shown in Fig. 1, *H. circularisquama* caused hemolysis of erythrocytes from various species to different extents. Among the species tested, rabbit erythrocyte showed the highest sensitivity. Guinea-pig erythrocytes were also highly sensitive but sheep, cattle and human erythrocytes were relatively insensitive against the hemolytic activity of *H. circularisquama*.

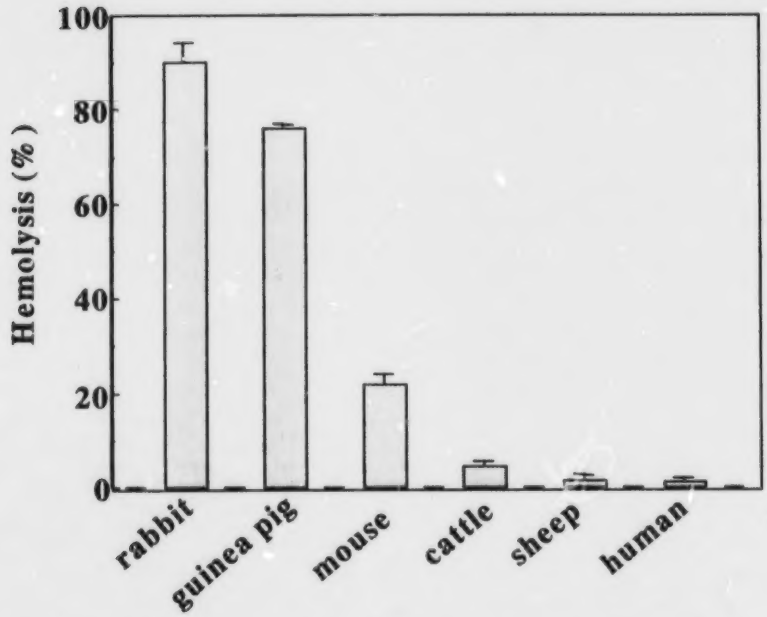

Fig. 1. Hemolytic activity of *Heterocapsa circularisquama* against erythrocytes from different animal species. *H. circularisquama* (final 10,000 cells mL^{-1}) were mixed with erythrocytes from rabbit, guinea-pig, mouse, cattle, sheep, or human and incubated for 5 h at 26°C. Hemolytic activity was assayed as described in the text. Each error bar represents ± 1SD.

Table 1. Effects of *H. circularisquama* and the ethanol extract on unfertilized eggs of pacific oyster. Unfertilized eggs (final 5,000 eggs mL^{-1}) were inoculated into 10^5 cells mL^{-1} of *H. circularisquama* cell suspension or the 2% ethanol extract in ESM medium. After the incubation at 26°C for 1, 2, and 3 h, the numbers of morphologically intact eggs in each sample were counted.

	Morphologically intact eggs (% of initial level)		
Incubation time	1 h	2 h	3 h
In *H. circularisquama* cell suspension (5 x 10^4 cells mL^{-1})	100 ± 0	92 ± 5	62 ± 3.0
In the ethanol extract (1%)	73 ± 4	5 ± 2	0 ± 0

The hemolytic activity of the culture supernatant prepared from *H. circularisquama* at late exponential growth phase was examined using rabbit erythrocytes. As shown in Fig. 2, the supernatant showed weak but significant hemolytic activity. The ultrasonic-ruptured cell

suspension prepared from the same *H. circularisquama* culture showed even less hemolytic activity than the supernatant (Fig. 2).

The presence of hemolytic agents in *H. circularisquama* was further examined by testing cell-free extract from the flagellate cells using 100% ethanol. Hemolytic activity was efficiently extracted into ethanol, and the ethanol extract showed strong hemolytic activity in a concentration-dependent manner at a concentration range at which ethanol alone had no hemolytic effect (Fig. 3). The hemolytic activity of the ethanol extract was relatively stable and no marked decrease in the activity was observed during a few weeks storage at -30°C.

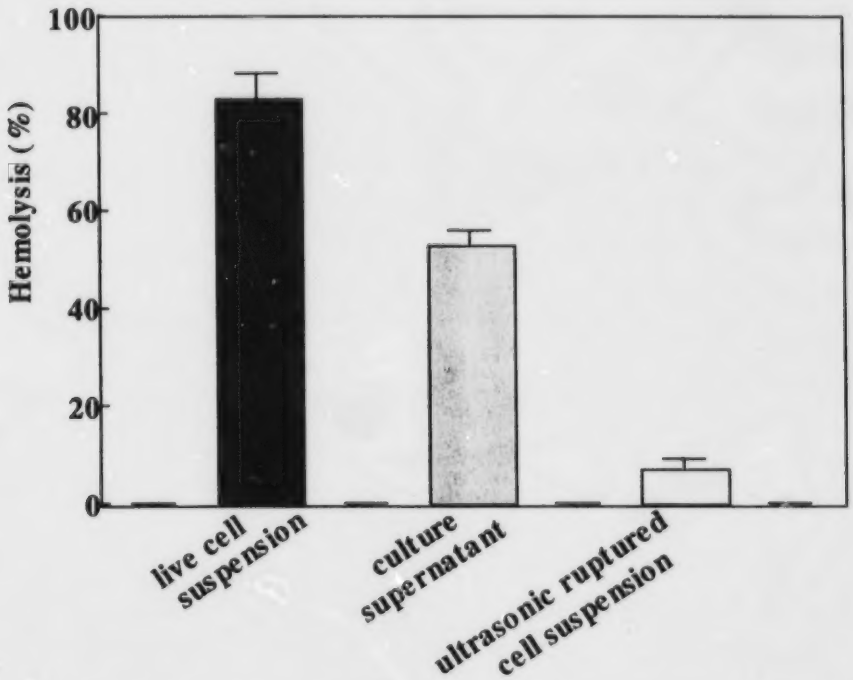

Fig. 2. Hemolytic activities of live cell suspension, culture supernatant, or ultrasonic ruptured cell suspension of *H. circularisquama*. Each sample equivalent to 10,000 cells mL^{-1} of live cell concentration was mixed with rabbit erythrocytes and incubated at 26°C for 5 h. Each error bar represents ± 1SD.

Comparison of hemolytic activities of toxic and less toxic strains of *H. circularisquama* and *Heterocapsa triquetra*

As shown in Fig. 4, the toxic strain of *H. circularisquama* showed stronger hemolytic activity than less toxic strain. Furthermore, *H. triquetra* showed no hemolytic activity toward rabbit erythrocytes, even at very high cell densities.

Toxic effects of *H. circularisquama* and its ethanol-extract on unfertilized eggs of Pacific oyster and microzooplankton rotifers (*Brachionus plicatilis*)

It has been reported that *H. circularisquama* showed toxic effects on unfertilized eggs of the Pacific oysters (*Crassostrea gigas*) in addition to mature bivalve mollusks (Matsuyama, 1999). The ethanol extract of *H. circularisquama* also caused similar changes on unfertilized eggs. In the presence of the 1% ethanol extract, such morphological changes proceeded even faster than in 5 x 10^4cells $mL^{-1}$of live flagellate cell suspension (Table 1). In addition, *H. circularisquama* showed lethal effects on microzooplankton rotifers (*Brachionus plicatilis*), and all the rotifers eventually died after being immobilized during the exposure to the high density of *H. circularisquama* (10^5 cells mL^{-1}) (Kim *et al.*, 2000). The ethanol extract also produced lethal effect on rotifers in a concentration dependent manner (Table 2).

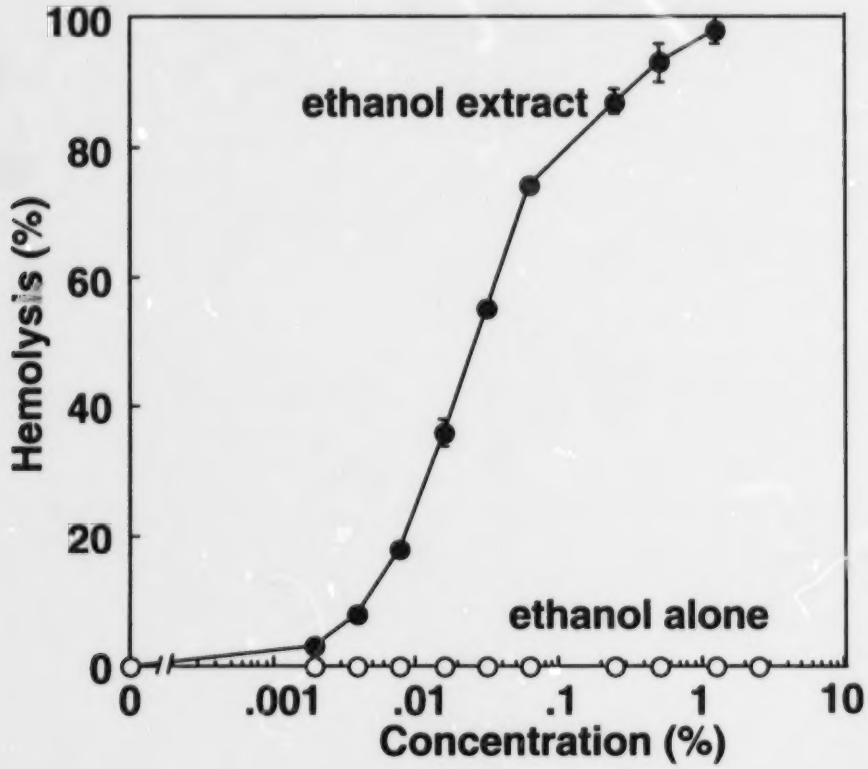

Fig. 3. Hemolytic activity of the ethanol extract of *H. circularisquama*. Rabbit erythrocytes were mixed with varying concentrations of the ethanol extract (●) or ethanol (○), and incubated at 26°C for 5 h. Each error bar represents ± 1SD.

Table 2. Effects of *H. circularisquama* and the ethanol extract on rotifer *Brachionus plicatilis*. Rotifers were inoculated into varying concentrations of *H. circularisquama* (1,000, 10,000, and 100,000 cells·mL^{-1}) or the ethanol extract (1.25, 2.5, 5, and 10%). After a 5 h incubation at 26°C, the number of viable rotifers in each sample was counted.

	Viability of rotifers (% of control)
In *H. circularisquama* cell suspension	
1,000 cellsl·mL^{-1}	80 ± 10
10,000 cells·mL^{-1}	65 ± 5
100,000 cells·mL^{-1}	0 ± 0
In the ethanol extract	
1.25%	90 ± 10
2.5%	60 ± 5
5%	30 ± 5
10%	0 ± 0

DISCUSSION

We found that the hemolytic activity of *H. circularisquama* is species specific. The sensitive erythrocytes are from rabbit, mouse, and guinea-pig, all of which have K^+ as the main intracellular cation. The resistant erythrocytes are from human, cattle, and sheep. Cattle and sheep erythrocytes have Na^+ as their main intracellular cation, while human erythrocytes have K^+ as their main intracellular cation (Habermann *et al.*, 1981). The species-specific hemolytic activity of *H. circularisquama* suggests the presence of certain toxins in this flagellate, which may act through a specific cellular receptors.

Species-specific hemolysis has been found in palytoxin, which is synthesized by corals of the family Palythoa (*Palythoa caribaearum*) and is the most potent animal toxin known (Habermann *et al.*, 1981; Habermann, 1989). Cattle and sheep erythrocytes, which are less sensitive to palytoxin, possess more Na^+ than K^+ as their intracellular cations. Although the reason is uncertain now, it has been reported that human erythrocytes, which have K^+ as their main intracellular cation, is highly resistant to palytoxin as compared to rabbit, guinea-pig, and mouse erythrocytes (Habermann *et al.*, 1981). Similar resistance of human erythrocytes against the hemolytic activity of *H. circularisquama* was observed as described above. Palytoxin appears to promote Na^+ influx and K^+ efflux from vertebrate cells (Ishida *et al.*, 1985; Habermann *et al.*, 1989). The influx of sodium promotes Na^+/Ca^{2+} exchange and depolarization, which in turn opens voltage-dependent Ca^{2+} channels. The resulting increase in intracellular Ca^{2+} concentration triggers numerous secondary pharmacological effects of palytoxin on contractile and secretary cells (Habermann, 1989). Although there is no available evidence indicating that *H. circularisquama* has palytoxin-like toxins right now, it has been reported that the symptoms observed in pearl oyster exposed to *H. circularisquama* were vigorous clapping, shrinkage of

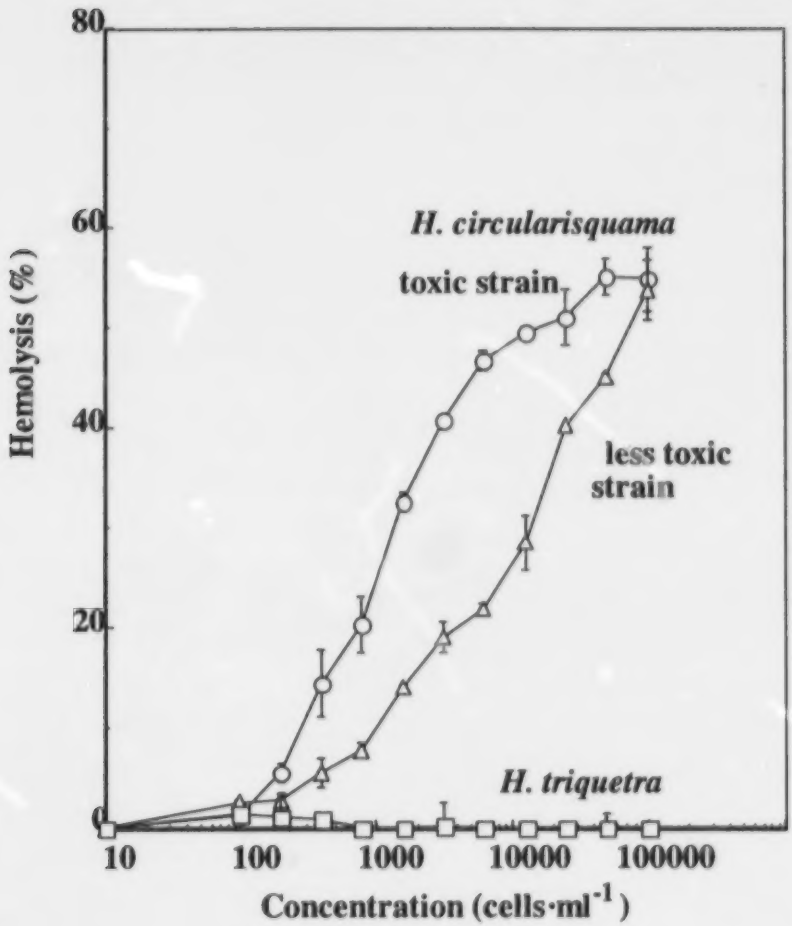

Fig. 4. Comparison of hemolytic activities of toxic (○) and less toxic (△) strains of *H. circularisquama* and *H. triquetra* (□). Increasing concentrations of each flagellate cells were mixed with rabbit erythrocytes and incubated for 5 h at 26°C. Each error bar represents ±1 SD.

their mantle edges and gills, and subsequent cardiac arrest, all of which are affected by Ca^{2+} ion flux (Nagai *et al.*, 1996). Furthermore, dramatic influx of Ca^{2+} have been observed in trochophore larva of short necked clams (*Ruditapes philippinarum*) after exposure to *H. circularisquama* (Matsuyama, 1999). These results suggest that the *H. circularisquama* toxin acts on erythrocyte membranes through an effect on ion channel, in a manner similar to that proposed for palytoxin.

The ethanol extract of *H. circularisquama* showed hemolytic activity as well as toxic effects on unfertilized eggs of the Pacific oyster and rotifers, suggesting that the toxic substances can be efficiently extracted into ethanol and such toxic compounds may be more stable in ethanol than in aqueous solution. In addition to the biochemical characterization of the ethanol extract, further

studies are required to clarify if the substances extracted in ethanol are actually involved in the toxic action of *H. circularisquama* on bivalves.

ACKNOWLEDGEMENTS

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THE SPATIAL VARIABILITY OF DOMOIC ACID CONCENTRATIONS IN RAZOR CLAMS AT KALALOCH BEACH, WASHINGTON

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Domoic acid levels in Washington State razor clams (*Siliqua patula*) have been extremely variable and unpredictable, resulting in emergency closures of harvest areas in 1991, 1998, and 1999. Information concerning toxin variability relative to sampling location is important in developing a reliable sampling plan for managing domoic acid outbreaks. In November 1998, record levels of domoic in razor clams (up to 297 ppm) were reported at Kalaloch Beach, Washington. Due to the long toxin retention time in these clams, a resource survey at Kalaloch conducted during the summer of 1999 presented an opportunity for the study of domoic acid levels as a function of tidal elevation and north-south beach location. From July 28-31, 1999 (during the summer low tides) razor clams were collected from six "east-west" transects at Kalaloch Beach, approximately 1.6 km apart. Each clam was individually analyzed for domoic acid in order to determine the distribution of toxin between (interspecific variability) and within (intraspecific variability) transects. While average domoic acid levels were similar between transects, toxin levels varied substantially among individual clam samples. The coefficient of variation among all samples was 122%, indicating that harvest closures based upon composite analyses of 6 clam samples could be in error. Here we recommend a sample size of razor clams for regulatory purposes that will result in domoic acid measurements more representative of total population toxin values.

DSP AND ASP TOXINS IN THE ESTUARY AND GULF OF ST. LAWRENCE: DISTRIBUTION AND CAUSATIVE ORGANISMS

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This study was initiated following the detection for the first time of DSP and ASP phycotoxins in shellfish (blue mussels and scallops) from the Magdalen Islands during the summer of 1998. Our objectives were 1) to determine if *Prorocentrum lima* may be the source of DSP in cultured mussels from the Magdalen Islands and 2) to evaluate the extent of the contamination by DSP and ASP of molluscs from different areas in Quebec. Our sampling, carried out at two cultured mussel sites in the Magdalen Islands, revealed the regular presence of *P. lima* fixed on the epibionts growing on the mussel socks. *P. lima* was frequently found in the digestive glands of the mussels, but apparently in concentrations too low to cause contamination during our study. In other regions of Quebec, 9.5% of the 252 shellfish analysed (mostly mussels) showed low levels of DTX toxins, with a maximum of 0.4 µg/g in the digestive gland. During the sampling conducted in the Magdalen Islands, we also identified, for the first time in Eastern Canada, another toxic microalga, *Prorocentrum mexicanum*. *P. mexicanum* is known to produce DSP-type toxins, but different from those produced by *P. lima*. These toxins were not measured during our study but could represent a risk that needs to be evaluated. Finally, elevated concentrations of ASP (up to 550 µg/g) were measured in the digestive glands of scallops from the Magdalen Islands. The source of these toxins and the importance of the contamination remain to be determined.

ASP IN THE SCALLOP *PECTEN MAXIMUS* - WHAT ARE THE ISSUES?

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ASP was first officially recorded in the scallop *Pecten maximus* in Scotland in 1998. In both 1999 and 2000 most Scottish scallop fishing grounds were closed to harvesting due to ASP contamination. A great deal of political and media interest followed, with intense debate on a number of issues. This presentation details the event and considers the possibility of a tiered monitoring system and the toxin content of adductor muscles. It describes research to estimate inter-animal and spatial variation in toxin concentration in different organs using scallops from several fishing grounds. It details a monitoring and regulatory strategy that uses information about inter-animal variability to limit the 'risk' to the consumer. It also poses questions regarding regulatory limits.

ACCUMULATION, DISTRIBUTION AND DEPURATION OF DOMOIC ACID BY *CHLAMYS* AND *MIZUHOPECTEN* SCALLOPS, WHEN FED BATCH CULTURED *PSEUDO-NITZSCHIA MULTISERIES*

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Pseudo-nitzschia multiseries at 8.7 – 19.6 x 10^4 cells mL^{-1}, was cultured in 500 L plastic bags under continuous full-spectrum light. *Mizuhopecten yessoensis*, *Chlamys hastata*, and *Chlamys rubida*, in triplicate trials, were fed to satiation with cultured algae that ranged in toxicity from 3.2 – 5.0 pg domoic acid (DA) per cell. After 0.5 h of feeding the DA in the viscera of *M. yessoensis*, *C. hastata* and *C. rubida* was 18.9, 23.9, and 35.4 $\mu g\ g^{-1}$, corresponding to 75%, 63%, 92% of the total DA uptake. The % of DA in the viscera, adductor, mantle, gills and gonad tissue of *M. yessoensis* remained relatively constant during the time of exposure (Fig. 1).

Fig. 1. Percentage of domoic acid accumulated in body parts of the scallop *Mizuhopecten yessoensis*

By comparison, the mean % toxin in the viscera of both *Chlamys* species declined with time of exposure. The decline in visceral toxicity was compensated in *C. rubida* by an increase on DA levels mainly in the mantle and gill tissue (Fig. 2). However, in *C. hastata* an increase was also observed in the adductor muscle (Fig. 3). The mean % toxin in the gonad tissue of *M. yessoensis*, *C. rubida* and *C. hastata* was relatively constant at 7.3, 5.3 and 7.5 % of the total toxin assimilated.

Fig. 2. Percentage of accumulated domoic acid in body parts of the scallop *Chlamys rubida*

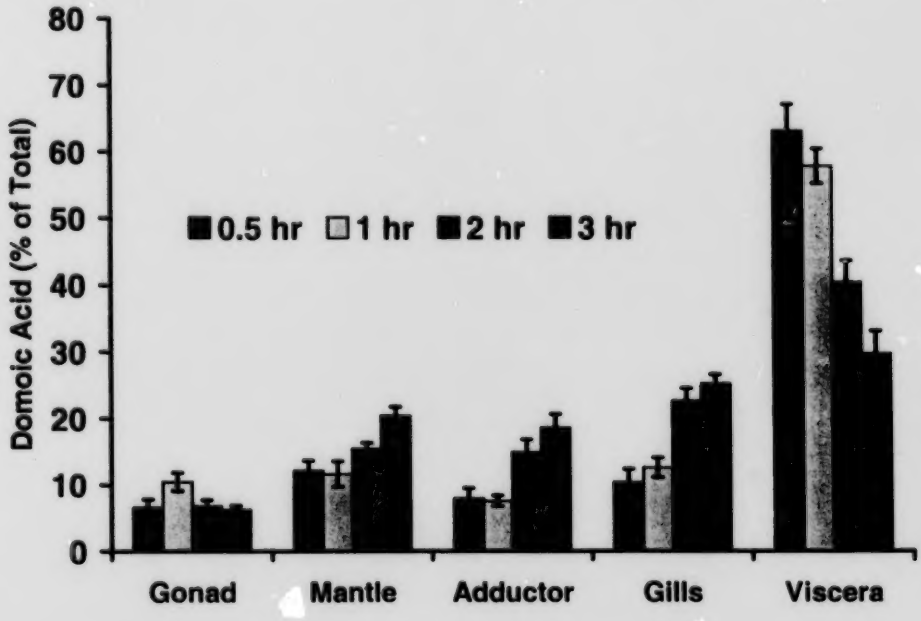

Fig. 3. Percentage of accumulated domoic acid in the body parts of the scallop *Chlamys hastata*

The intoxicated scallops, subsequently maintained in flowing seawater for up to 72 h, provided rates of depuration in the visceral tissue best expressed by the equation, $Y = ae^{-bx}$, with b values of 0.18, 0.16 and 0.06 for *M. yessoensis*, *C. rubida* and *C. hastata*, respectively. The considerably lower rate of clearance of toxin from *C. hastata* reflected the greater uptake into the adductor muscle, which resulted in a compromised motor response.

POSTER SESSION

KALALOCH BEACH: A HOTSPOT FOR DOMOIC ACID IN WASHINGTON STATE

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After the appearance of domoic acid (DA) in razor clams on the Washington outer coast in 1991, the state commenced monitoring operations for the neurotoxin in shellfish. Prior to 1991, shellfish had only been tested for the presence of paralytic shellfish toxins. In addition, the coast of the state from the mouth of the Columbia river to Dungeness spit in the Strait of Juan de Fuca was closed to recreational shellfish harvesting from April through October of each year due to measurements of paralytic shellfish toxins. Razor clams were exempt from that ban. Presently, the blanket closure is still in effect but razor clams are now included. Since testing for DA began in 1991, consistently higher levels of the toxin have been seen in razor collected at Kalaloch beach than other beaches on the Washington coast when there was a conspicuous DA outbreak. Recent studies have documented that the elevated levels of DA in shellfish at Kalaloch beach are preceded by high levels of particulate DA in seawater and high numbers of toxin-producing *Pseudo-nitzschia*. It has been theorized that an offshore population of *Pseudo-nitzschia* may be advected to the coast by yet unknown physical processes resulting in the toxification of razor clams with the highest levels of DA routinely observed at Kalaloch beach.

DETECTION METHODS FOR DOMOIC ACID IN SEAWATER

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There are several methods used to detect domoic acid in seawater and shellfish such as high performance liquid chromatography, a receptor binding assay, and an immunoassay. The receptor binding assay (RBA) measures the competitive displacement of radiolabeled kainic acid bound to a cloned glutamate receptor (GluR6) by domoic acid in a sample. The immunoassay measures the competition of sample domoic acid for antibody (indirect competitive enzyme-linked immunosorbent assay (ELISA)). Due to its sensitivity and cost effectiveness, the receptor-binding assay is commonly used; however, the ELISA shows promise as an alternative method. The primary disadvantage of each assay is that polyclonal antibody supply is limited (ELISA) and receptor isolation is time consuming (RBA). The purpose of this poster is to compare assay steps, assay qualities such as sensitivity and robustness, and results of DA detection in seawater of common samples. The seawater samples used for the comparison were collected from a 1998 harmful algal bloom off the coast of central California in which over 70 California sea lions were found stranded due to neurological damage caused by domoic acid poisoning. This was the first demonstration of domoic acid transfer through the food chain to a marine mammal. *Pseudo-nitzschia multiseries* and *P. australis* were the dominant toxin producing species. The high levels of domoic acid in this bloom provided an excellent opportunity for the analysis of domoic acid in seawater. Sample preparation is the same for both of the analyses. One liter of seawater was filtered on to an HA filter (45 mm diameter). Thereafter, the filters were cut and placed into a 15 mL Falcon tube and 5 mL of distilled water was added. The sample was then sonicated and centrifuged. A comparison of the two assays showed that the ELISA is more sensitive whereas the receptor binding assay has a larger working range. The limit of quantitation, the detection limit, and working range for the RBA are 5 ng/mL, 3.1 ng/mL and 5-100 ng/mL respectively. The limit of quantitation, the detection limit, and the working range for the ELISA are 0.15 ng/mL, 0.01 ng/mL and 0.15-15 ng/mL, respectively. The receptor binding assay and the ELISA yield statistically equivalent results for domoic acid detection in seawater. However, on average the ELISA DA values were about 60% less than the RBA values. Further work with the ELISA dilutions for samples with DA levels above 6000 ng/L could possibly result in better agreement between the two assays.

MARINE MAMMAL SUSCEPTIBILITY TO DOMOIC ACID POISONING

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During May and June of 1998, over 400 California sea lions (*Zalopus californianus*) died and displayed signs of neurological dysfunction along the central California coast. Concurrent with this event, a bloom of *Pseudo–nitzschia australis* (a pennate diatom that produces the neurotoxin, domoic acid) was observed in Monterey Bay, California. Domoic acid (DA) was detected in planktivorous fish and sea lion body fluids at that time. This study demonstrates that DA binds to isolated nerve preparations of California sea lion with similar affinity to that reported for nerve preparations of rat and frog. Analysis of receptor binding of tritiated kainic acid (KA), a toxin with similar binding affinities as DA, to sea lion brain, illustrates saturable specific binding and competition of specific binding by non-radioactive DA. This study also demonstrates that DA binds with high affinity to isolated nerve preparations of other marine mammals, including gray whale (*Eschrichtius robustus*), northern fur seal (*Callorhinus ursinus*), sea otter (*Enhydra lutris*), and northern elephant seal (*Mirounga angustirostris*). These results demonstrate the specific binding of DA to brain tissue of marine mammals and support the hypothesis that the exposure of California sea lions to DA in May and June of 1998 was a factor in their death and neurological dysfunction.

DISTRIBUTION OF EPIPHYTIC BACTERIA ON *PSEUDO-NITZSCHIA MULTISERIES* CELLS IN CULTURE

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The toxicity of the domoic-acid-producing pennate diatom *Pseudo-nitzschia multiseries* is enhanced by association with bacteria. Yet, apart from the initial molecular studies nearly a decade ago, the diversity of bacteria in cultures of *P. multiseries* has not been explored. The goal of this study was to examine the frequency and distribution pattern of epiphytic bacteria growing on cultured cells of *P. multiseries*. Diatoms were grown in flasks of f/2 medium for 31 d. Every 5-12 d, 5 one-mL subsamples were transferred to a filtration apparatus and grown undisturbed for another 2 d in order to maintain natural associations between the diatom host and its bacterial epiphytes. The subsamples were then gently prepared for SEM examination. From 15-40% of the diatom cells had attached bacteria. Most diatom cells carried only 1-5 bacteria, although some had up to 74 bacteria. Even in late stationary phase, many diatom cells were bacteria-free. A morphologically diverse bacterial flora was observed attached to the host diatoms, including: 1) stalked bacteria (Fig. 1), 2) rod-shaped bacteria attached by their narrow side, 3) rods attached along their long side, and 4) cocci. Stalked bacteria were found most commonly on the girdle side of the diatom host, in the girdle band junctions (Fig. 1). They also were also attached to the raphe and striae. Rod-shaped bacteria resting on their long side were found most commonly on the valve face, on the striae. These preliminary results indicate that cultured diatoms co-exist with a diverse bacterial epiphytic flora, having a specific distribution pattern and frequency. Their effect on domoic acid production has yet to be determined.

Fig. 1. A short colony of three *Pseudo-nitzschia* cells (the two bottom cells are recently divided) carries a number of bacterial epibionts. The morphotype of bacterium shown (M-1) is attached most commonly to the diatom host at the girdle band junctions and near the raphe, in areas where cell organic matter may be seen oozing from the host. This SEM image is a montage of 3 images.

GROWTH STIMULATION OF *ALEXANDRIUM TAMARENSE* CULTURES BY HUMIC SUBSTANCES FROM THE MANICOUAGAN RIVER (EASTERN CANADA)

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In the St. Lawrence Estuary, annually recurrent blooms of the toxic dinoflagellate *Alexandrium tamarense* are associated with brackish waters. Riverine inputs are suspected to favour bloom development by increasing water column stability and/or providing growth stimulants such as humic substances (HS). A 17-d culture experiment was conducted to evaluate the importance of HS from the Manigouagan River as growth factors for *A. tamarense*. Non-axenic cultures were exposed to four (HS) extracts from three different sources: humic and fulvic acids isolated from the Manicouagan River, humic acids from the Suwannee River and desalted alkaline soil extract. For each extract, four concentrations were tested as supplements to the non-limiting artificial Keller (AK) medium. HS additions from all sources significantly enhanced the overall growth rates relative to the control treatment. The highest growth rates (μ = 0.53±0.05 d^{-1}) were sustained during half of the experiment. The growth response of *A. tamarense* to HS additions reached its maximum after four days. The exponential growth phase lasted for four more days. The HS concentration, measured by UV-spectrophotometry, remained constant throughout the exponential phase, suggesting that HS act mainly as growth promoters in this experiment. However, a significant reduction of the HS concentration was observed at the end of the growth phase when the nitrate and phosphate concentrations were still high (783±10 μM and 3.7±0.1 μM, respectively). Dose-response curves indicate that HS can increase the growth rate of *A. tamarense* even at low concentrations such as those encountered in estuaries. Our results support the hypothesis that HS in the Manicouagan River plume can stimulate the development of toxic dinoflagellate blooms.

SEMI-CONTINUOUS CULTURE OF TOXIC *ALEXANDRIUM TAMARENSE* IN LARGE-SCALE PHOTOBIOREACTORS

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INTRODUCTION

Production of toxigenic phytoplankton in quantities sufficient for shellfish experimentation, or for extraction of pure toxins for preparation of standards and reference materials, has proved notoriously challenging. Critical requirements are that a high and relatively constant cell toxicity be maintained, with minimal variation in toxin quantity and composition. Toxin content of *Alexandrium* spp. is known to vary greatly under different growth conditions (age of culture, temperature and nitrogen and phosphorus availability), whereas the toxin profile is generally a more conservative feature for a given dinoflagellate isolate (reviewed by Cembella, 1998). However, marked changes in the relative abundance of individual toxins were documented in semi-continuous cultures of *Alexandrium fundyense* (Anderson *et al.*, 1990a). Traditional methods usually entail batch culture in 20-L or 36-L carboys, which require staggered scaled-up cultures to ensure a daily supply of cells in the same growth phase. This approach is labor-intensive, and requires considerable space in expensive, environmentally controlled growth chambers.

To meet these demands, novel photobioreactors (200-L and 500-L capacities) invented at the Institute for Marine Biosciences (IMB), were employed to grow semi-continuous cultures of *Alexandrium tamarense* (isolate Pr18b), a producer of paralytic shellfish poisoning (PSP) toxins. Live cells were either fed to softshell clams (*Mya arenaria*) during toxification studies or were harvested by centrifuge for toxin extraction and preparation of standards for the IMB Certified Reference Material Program (CRMP).

MATERIALS AND METHODS

Alexandrium tamarense (a high-toxicity isolate, Pr18b, from the lower St. Lawrence Estuary, Québec) was cultured at IMB's Aquaculture Research Station, Sambro, NS, in a 200-L photobioreactor in semi-continuous mode for 50 d. The photobioreactor was a closed system vessel, illuminated internally to give an initial photon flux density of 1500 μmol m^{-2} s^{-1}, and fitted for semi-continuous harvesting of the algal cultures. Details of the photobioreactor design cannot be released at this time, as a provisory patent application and commercialization of this technology are in progress. The vessel was inoculated with 30 L of culture harvested in early exponential growth phase (2900 cells mL^{-1}) from a 36 L carboy. The culture medium of 1 μm cartridge-filtered, pasteurized seawater (salinity = 30 ppt) with L1 nutrients (Guillard and Hargraves, 1993), modified by the addition of ammonium (R.R.L. Guillard, Bigelow Laboratory

for Ocean Sciences, West Boothbay Harbor, Maine 04575, USA, personal communication; 883 μM nitrate, NO_3^-, 50 μM NH_4^+, 36 μM phosphate, PO_4^{-3}) was continuously aerated to ensure a homogeneous cell distribution. Temperature was held at ~16°C; pH was maintained initially at ~8.2 and later at 8.6 by titration with CO_2 on demand. Irradiance was increased gradually from 25% to 100% of maximum photon flux density during the initial, batch phase of the run. The photoperiod was 14 h L:10 h D. Approximately 40% of the culture was harvested via a peristaltic pump (d 27, 36, 41, and 45) at cell densities between 4000 and 11,600 mL^{-1}, and 100% was harvested at 50 d. Harvests, pH readings and sampling for nutrients were conducted at approximately the same point in the photocycle. The photobioreactor was refilled to full capacity (200 L) with pasteurized, nutrient-enriched seawater immediately after each harvest. Culture harvested for toxin production was pumped through a continuous-flow centrifuge. Biomass (algal paste) for preparative extraction of PSP toxins was weighed and stored at –80°C.

Previous studies indicate that the toxicity of *A. tamarense* is maximal when the N:P ratio in the culture medium is high, i.e. under N-replete and P-limited conditions (Anderson *et al.*, 1990b; MacIntyre *et al.*, 1997). Thus, to ensure that the culture produced highly toxic cells, the medium was supplemented with ammonium and nitrate on d 17 (single dose calculated for 80 L) and immediately after harvests on d 36, 41 and 45 (2x the modified L1 concentrations calculated for the 90 - 100 L replacement volumes).

Samples were collected from the photobioreactor every 1-2 d to measure cell density and size (Coulter-Multisizer), soluble nutrients (NO_3^-, PO_4^{-3}), cellular carbon and nitrogen (CHN Analyzer), and toxin content. Potential contamination was also monitored by microscopic observation. Growth rates (k, div. d^{-1}) were calculated as $k = [\ln (N_{t1}/N_{t2})/ (t_2 - t_1)]/\ln 2$, where N_{t1} and N_{t2} are cell densities at the beginning and end of each time interval. For toxin extraction, cells were concentrated on a 20 μm-mesh sieve, collected by centrifugation (at 4000 x *g* and 5°C for 20 min) and stored at –80°C until extraction. Toxins were extracted by sonication in 0.03M acetic acid, filtered (Gelman Acrodisc 2 μm syringe filters) and individually quantified by high-performance liquid chromatography with post-column oxidation and fluorescence detection (HPLC-FD) following Oshima (1995), using toxin standards produced by CRMP. Toxin-specific conversion factors (determined by Oshima 1995) were used to calculate toxicity [in μg saxitoxin equivalents (STXeq) $cell^{-1}$] from molar concentrations of individual toxins. Dissolved nutrients (NO_3^-, PO_4^{-3}) were measured by filtering 30 mL samples through Whatman GF/F filters; the filtrate was stored at -80°C until analyzed following methods described by Strain and Clement (1996). Ammonium concentrations were not determined. Phosphate utilization rates by *Alexandrium* cells were calculated by assuming a linear decline in concentration in the medium over the selected intervals.

RESULTS

The experiment described was conducted in a 200-L photobioreactor, and yielded 5 harvests [total toxin production = 0.33 g STXeq (Table 1); total algal biomass = 129 g packed wet weight]. The trial was terminated on d 50 due to contamination by a 2 μm zooflagellate. Harvestable biomass of toxic cells with densities > 3000 cells mL^{-1} was obtained in 8 of 12 trials conducted in photobioreactors to date. Following an initial lag phase, the culture achieved relatively high division rates (up to 0.26 d^{-1}, although growth declined in the last 10 d) (Table 2),

and produced cells of consistently high toxicity at harvest (90 to 113 pg STXeq $cell^{-1}$; toxin content averaging 462 fmol $cell^{-1}$; Figs. 1b, 1c). This was accomplished by maintaining excess N (~1000 μM NO_3^-) and limiting the available PO_4^{-3} (2 - 16 μM) (Figs. 1b, 1d). Growth rates reported for this strain in batch or semi-continuous cultures averaged 0.3 d^{-1} (MacIntyre *et al.*, 1997). Mean toxicities achieved previously are typically lower, at 60 to 65 pg STXeq $cell^{-1}$ (MacQuarrie, unpublished data; MacIntyre *et al.*, 1997). *Alexandrium* cells consistently showed rapid PO_4^{-3} uptake rates (mean = 0.17 pmol $cell^{-1}$ d^{-1}, Table 3). Cellular toxin content was inversely related to PO_4^{-3} concentrations and generally increased below 5-8 μM, except in one case (Fig. 1b). Nitrate, supplied in excess, had no effect on toxin content.

The toxin profile remained relatively constant and comparable to that obtained in 20-L carboys (Fig. 2), regardless of the fluctuations in PO_4^{-3} content in the medium. Relative proportions of the principal toxins produced by strain Pr18b in the photobioreactor, the N-sulfocarbamoyl toxin C2, gonyautoxins GTX4, GTX3, neosaxitoxin (NEO) and STX, averaged 66.8, 1.3, 7.9, 18.2 and 5.5% molar, respectively. Improved growth performance was noted at high pH (8.6) relative to that commonly used for culturing marine phytoplankton (7.8 - 8.2) (Fig. 1c). Stable pH was achieved after d 31 by increasing aeration to improve mixing.

Cell size was somewhat reduced at the time of inoculation (mean equivalent spherical diameter, ESD = 29.6 μm) but remained constant (mean ESD = 34.5 μm; SE = 0.15) for the duration of the trial (Fig. 3). The C/N molar ratio also remained constant (mean = 6.25; range = 5.82 to 6.53) (Fig. 3) and equal to reported values for this isolate under N-replete conditions (MacIntyre *et al.*, 1997). Not surprisingly, greatest changes in all cellular parameters measured (toxin and N content, C/N ratio and cell size) occurred in the first few d following inoculation of the photobioreactor, and presumably reflect the change in culture conditions.

Table 1. Harvest and PSP production data for *Alexandrium tamarense* grown in a 200-L photobioreactor.

Days	Harvest volume (L)	Harvest cell density (cells mL^{-1})	Cells harvested (millions)	Toxin production mg STXeq (mmol)
27	80	4030	322.4	30.3 (150.4)
36	90	8370	753.3	71.5 (379.1)
41	90	11,588	1042.9	106.2 (389.2)
45	100	8548	854.8	96.2 (447.2)
50	200	1542	308.4	27.6 (136.9)
Totals	560	-	3281.8	331.8 (1502.8)

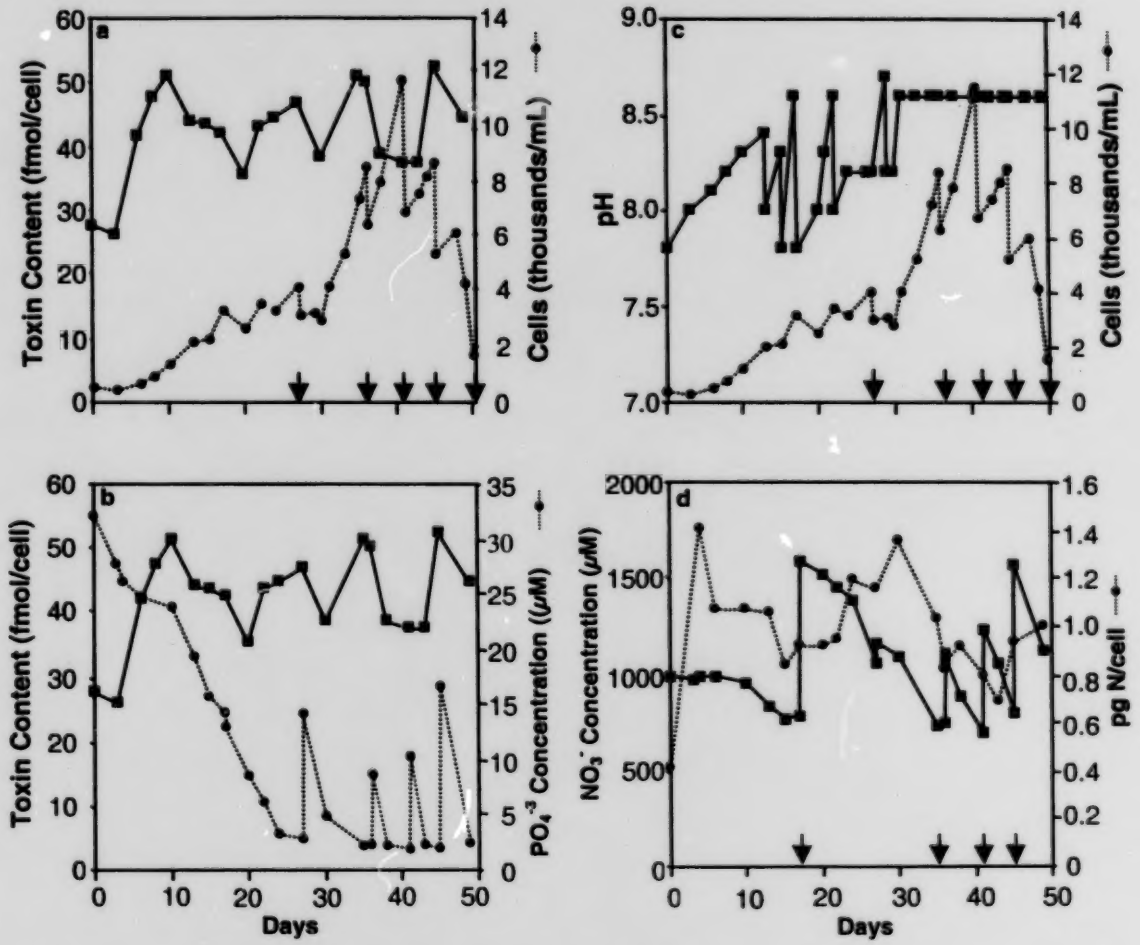

Fig. 1. Temporal patterns of *Alexandrium tamarense* cell density in relation to: a) cellular toxin content, and c) pH; relationship between b) cellular toxin content and phosphate (PO_4^{-3}) concentration in the medium, and d) nitrate (NO_3^-) concentration in the medium and cellular nitrogen content; ↓timing of N spikes (NO_3^- + NH_4^+; see Methods); ↓timing of harvests (Table 1).

Table 2. Growth rates of *Alexandrium tamarense* (Pr18b).

Interval (d)	Divisions d^{-1}
3 – 27	0.16
30 – 36	0.26
36 – 41	0.18
41 – 45	0.09

Table 3. Phosphate uptake rate of *Alexandrium tamarense* (Pr18b).

Interval (d)	PO_4^{-3} (pmol $cell^{-1}$ d^{-1})
3 – 27	0.48
27 – 30	1.10
36 – 38	4.82
41 – 43	0.60
45 – 49	0.78

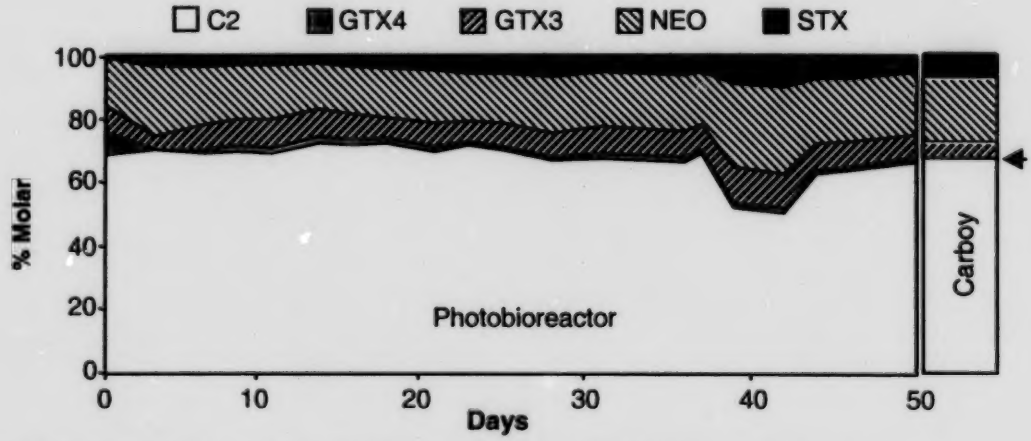

Fig. 2. Toxin composition of *Alexandrium tamarense* cells (Pr18b) produced in the photobioreactor. Mean toxin composition obtained in 20-L carboy cultures (MacQuarrie, unpublished data) is shown for comparison (◄ traces of GTX4 were detected in carboy cultures).

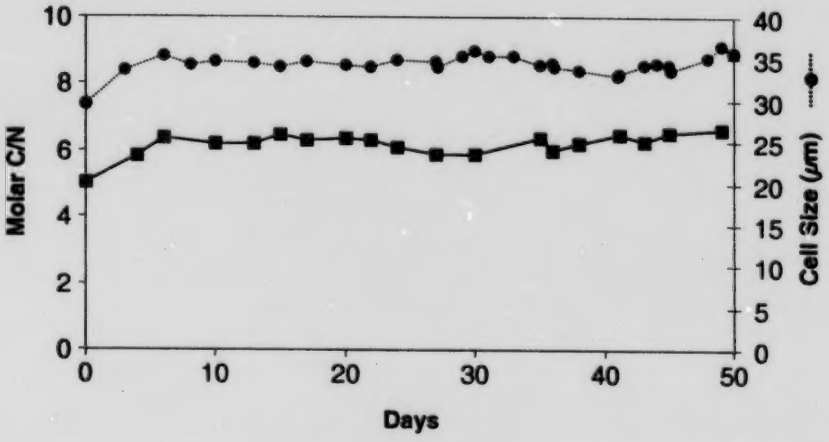

Fig. 3. Molar C/N ratio and mean cell size (equivalent spherical diameter) of *Alexandrium tamarense* (Pr18b).

DISCUSSION

The present study demonstrates that significant quantities of the dinoflagellate *A. tamarense* can be produced using our photobioreactor design. High cell toxicity was maintained throughout the trial with toxicity levels equal to, or exceeding those reported for smaller scale, batch and semi-continuous cultures of this isolate. The relative proportion of individual PSP toxins remained essentially constant and was comparable to that routinely obtained in small-scale batch cultures (20-L carboys). Therefore, biomass of high toxicity and consistent toxin composition can be generated in a relatively facile manner with considerable saving in labor compared with traditional carboy culture protocols.

Relatively high cellular toxicity levels were achieved in a medium containing excess nitrogen (~1000 μM NO_3^-) and low phosphorus concentrations (2.0 – 16.7 μM PO_4^{-3}). These results support those of previous studies, which demonstrated that P-limitation enhances toxin production and cellular toxin content in *Alexandrium* species (Boyer *et al.*, 1987; Anderson *et al.*, 1990b; MacIntyre *et al.*, 1997), and implicate phosphorus in the regulation of toxin metabolism (Flynn *et al.*, 1994). Anderson *et al.* (1990b) suggested that P-limitation may reduce the competitive demand for arginine, a free amino acid, in P-dependent metabolic pathways, allowing excess arginine to be used as a precursor in PSP-toxin synthesis. However, Flynn *et al.* (1994) concluded that the availability of arginine alone was not the controlling factor in the toxicity of *A. minutum*. In the present study, *A. tamarense* cells showed very rapid uptake of PO_4^{-3} and relatively slow NO_3^- uptake. MacIntyre *et al.* (1990) also demonstrated that N-limitation forces *A. tamarense* cultures into stationary phase and results in significant decreases in toxin production. Toxin content per cell of *Alexandrium* spp. in batch culture is typically highest in mid-exponential growth phase, and declines in stationary phase (except when P-limitation causes cessation of growth), although the causes of this decline with age remain speculative (reviewed by Cembella, 1998). These findings led us to use extremely high N:P

ratios in the photobioreactor and to maintain cells in exponential growth. Although this strategy proved successful in producing highly toxic cells, future efforts should attempt to further enhance population growth rates while maintaining high toxin cell content and thereby maximize toxin production (fmol toxin per unit volume of culture).

The relatively long initial lag phase following inoculation may be partially attributed to fluctuations in pH (7.8 to 8.7) of the medium during this start-up period. The pH in the photobioreactors was controlled by titration of CO_2 via the air supply. Initially, the aeration of the system was maintained at a minimal flow so as not to disrupt the *A. tamarense* cells. The low rate of aeration resulted in inadequate mixing thus preventing the pH from being maintained at constant levels in the system. After observing that growth rates were consistently high during periods when the pH was at relatively high levels (~8.5), aeration was increased and the culture was maintained at pH 8.6. Optimum growth performance of the culture at this pH supports the results of Siu *et al.* (1997), who demonstrated that growth rates of *A. catenella* (isolate from Hong Kong waters) were highly sensitive to pH changes and were highest at pH 8.5 (range tested = 6.5 to 9.0, at 0.5 pH intervals). Further investigation is needed to determine the optimum pH for growth of *A. tamarense* isolates.

Further improvements of the pasteurized water supply system and rate of nutrient delivery (work in progress) are expected to result in even higher, sustained cell and toxin production rates. This photobioreactor has also been successfully used to routinely produce non-toxic algae for feeding bivalve spat (at IMB) and for "greening" water during the early life stages of finfish (at IMB and a commercial facility).

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EFFECTS OF ANTIBIOTIC TREATMENTS ON THE TOXIGENICITY OF *ALEXANDRIUM TAMARENSE* IN BATCH CULTURES

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A series of experiments was carried out to monitor how the dynamics of bacterial growth impact the growth and toxin production of the toxic dinoflagellate *Alexandrium tamarense* ATCI01 and determine the effects of antibiotics on this microalgae. Treated with different doses of antibiotics, there were dramatically different responses to the growth and toxin production of ATCI01. When the antibiotics (Penicillin-G and Streptomycin) were added to a final concentration of 100 μg/mL in the medium, algal cells number and total toxin production were enhanced markedly compared with the control groups. When the final concentration was 500 μg/mL, growth of algal cells was inhibited and then after a few days it recovered and reached a maximum on d 14. However, if the final concentration was 1000 μg/mL, algal cells were killed completely. The positive effect of antibiotics on the growth and toxin production in the axenic cultures showed that the antibiotics could not only inhibit the growth of bacteria, which make more nutrients available for algal cells, but also the physiological or genetic characteristics of the algal cells were possibly affected by the antibiotics.

EFFECTS OF BIOSYNTHETIC PRECURSORS ON THE TOXIGENECITY OF *ALEXANDRIUM TAMARENSE*

Pilong Li and Dennis P. Hsieh

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A toxigenic strain of *Alexandrium tamarense* (ATCI01) was found to produce C2 toxin (C2T), one of the paralytic shellfish toxins (PST), predominantly. This strain is being used to produce isotope-labeled C2T for research and monitoring purposes. Effects of different levels of PST biosynthetic precursors on toxigenicity of ATCI01 in natural seawater K-medium in 50 mL batch cultures were studied for the incorporation of isotope labels from the precursors into C2T. The precursors included nitrate and bicarbonate as general precursors and acetate and arginine as committed precursors. The growth and toxin production of ATCI01 were monitored by cell counting and by HPLC-FLD, respectively. Nitrate, bicarbonate and arginine enhanced toxin production but had no significant effects on the growth. Acetate did not promote toxin production but increased the toxin content of algal cells. These results have provided useful information for the design of cultures that will efficiently incorporate isotope-labels from the precursors into C2T. (The study is supported by the Hong Kong University Grant Council and the Research Ground Council.)

DOES *ALEXANDRIUM FUNDYENSE* IN THE BAY OF FUNDY MIGRATE VERTICALLY?

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A number of dinoflagellates have demonstrated a nocturnal downward migration in order to utilize nutrients and growth-promoting substances. The organism *Alexandrium fundyense* is responsible for producing paralytic shellfish toxins (PST) in the Bay of Fundy resulting in many shellfish areas being closed to harvesting for a period of time each year. A study of *A. fundyense* in the Bay of Fundy was conducted east of Grand Manan Island over a period of 54 h (10:00 h, July 27-16.00 h, July 29) where samples were collected at 3 h intervals from the surface and depths of 5 m, 10 m, 20 m, 30 m, 50 m, and 90 m. Analyses included dominant phytoplankton species with particular emphasis on total *A. fundyense* concentrations which included its various life cycle stages – fusing cells, duplets, planozygotes and newly formed resting cysts. Highest concentrations of total *A. fundyense* cells were detected in 15 of the 19 surface samplings with the highest numbers detected at 10:00 h. The remaining four samplings had highest concentrations detected at 5 m and all were between the hours of 10:00 h and 16:00 h. Surface concentrations ranged from 8.88×10^4 to 1.65×10^5 cells L^{-1}. Although cells were observed throughout the water column, *A. fundyense* numbers decreased with depth throughout the study and numbers at the 90 m depth ranged from 100-1900 cells L^{-1}. Similarly, planozygotes were observed throughout the water column with concentrations greatest in surface samples with the highest number (2.6×10^4 cells L^{-1}) observed at 13:00 h. Duplets were observed to a depth of 20 m but the majority were detected in the surface waters with no duplets detected at depths of 50 and 90 m. Highest numbers of duplets were 9.46×10^4 cells L^{-1} at 07:00 h. Numbers of fusing cells were significantly lower that those for duplets although the maximum density 5.11×10^3 cells L^{-1} was also detected at the surface. Newly formed resting cysts were also observed during the study, but only at depths of 50 and 90 m. Concentrations ranged from 20-300 cells L^{-1}. Mean surface and 90 m temperatures were 12.1°C and 7.9°C, respectively. Salinities ranged from 31.0-32.9 psu. Other species observed during the study included *Scrippsiella trochoidea*, *Mesodinium rubrum*, *Ptychocylis* sp., *Helicostomella* sp., *Favella* sp., and various other tintinnid species. Further research is required to determine why the Bay of Fundy *A. fundyense* strain does not migrate vertically. Attempts to reconstruct the study and do further analyses have been unsuccessful as a result of the extremely low concentrations in recent years.

DYNAMICS OF *ALEXANDRIUM CATENELLA* BLOOMS IN QUARTERMASTER HARBOR, WASHINGTON

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Alexandrium catenella, that causes paralytic shellfish poisoning (PSP) in Puget Sound, WA, was studied in Quartermaster Harbor, a small, semi-enclosed, seasonally stratified bay in central Puget Sound to determine the effects of biological, chemical, and physical factors on the formation, maintenance, and decline of blooms. A shore station was sampled near mid-bay 1-3 times per week from May to September 1998 to determine the environmental conditions of the surface layer in the bay during the optimal growing season for this species. Additionally, three cruises occupied a station at 20 m depth in 1997 and 1998 to study the diel migration pattern of the *Alexandrium* population and to estimate growth and mortality (from grazing) rates of the total dinoflagellate population from dilution experiments. Estimates of tidal flushing were made by calculating the volumes of the bay at various tide heights and the volumes of typical tidal prisms for mean, neap, and spring tide ranges. PSP concentrations in caged mussels at 1 m depth exceeded 80 μg 100 g^{-1} mussel meat twice during 1997 and 1998, both times following the occurrence of *A. catenella* concentrations > 10,000 cells L^{-1}. The species was present continuously from late May through September 1998, blooming in July in surface water. Over this time interval surface temperatures varied from 14-20 °C; nitrate levels remained near-zero; phosphate and silicate were low, but measurable; and salinity ranged from 25-30 psu. Thermal stratification beginning in late spring led to the development of a shallow, low-nutrient surface layer (upper 1-3 m), a distinct pycnocline (3-5 m depth), and a deeper, nutrient-replete layer (5-20 m). During the July 1998 cruise strong spring tidal cycles eroded the pycnocline and mixed the water column within 24 h. This disrupted the distribution of *A. catenella* and contributed to bloom decline. The diel vertical migration pattern of *A. catenella*, studied in 1997 and 1998, was limited to the upper 10 m of the water column with migration rates varying from 0.4 - 2.0 m h^{-1}. Maximal *A. catenella* cell numbers varied from < 10,000 - > 60,000 cells L^{-1}, but the phytoplankton assemblage was dominated by large diatoms which were present at > 500,000 cells L^{-1}. Dilution experiments in 1998 indicated that the total dinoflagellate population grew at about the same rate as the diatom population but was grazed more heavily by microzooplankton. If estimated loss due to tidal flushing of the population is added to grazing rate, the dinoflagellate population growth rate was less than the combined loss rate, but growth still exceeded loss for the diatoms during spring tidal exchange periods. However, during mean or neap tidal periods, population growth rate was higher than loss rate for both dinoflagellates and diatoms. Assuming that the results from the overall dinoflagellate population are applicable to *A. catenella*, it appears that when water temperature exceeds 13 °C, permitting active *A. catenella* growth, blooms are rarely generated and then only for a limited period each summer. A combination of biological, chemical, and physical factors can lead to decline of the bloom within the bay and prevent an accumulation of PSP in the shellfish.

A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION FOR POLYAMINES IN THE PSP-PRODUCING ALGA *ALEXANDRIUM MINUTUM*

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A HPLC procedure with C_{18} reversed-phase column for determination of 5 polyamines in the toxic dinoflagellate *Alexandrium minutum* T1 was developed. The 6% PCA solution was used to extract the polyamines in the algal cells. The optimal mobile system was methanol-water (50:50, v/v) for 0.5 min, methanol-water (50:50 to 75:25, v/v) over 6.5 min, and then methanol-water (75:20, v/v) for 5 min. Five polyamines putrescine (Put), cadaverine (Cad), spermine (Spm), spermidine (Spd), and norspermidine (Norspd) were clearly separated. The standard curve of each polyamine was linear in the range of 0.02-3 μg. The detected limit for each polyamines was 0.01 μg. Applying this method, the free and conjugated polyamines extracted from *A. minutum* T1 were determined. Both free and conjugated polyamines contained above 5 polyamines. During the growth circle of *A. minutum* T1, the level of Norspd and Put in the free polyamines were the highest at 3-d culture. Put and Cad were the major components in the conjugated polyamines. The amount of conjugated amines was higher than that of free amines since the exponential phase of *A. minutum* T1.

PARALYTIC SHELLFISH POISONING IN PUGET SOUND: A HISTORICAL PERSPECTIVE

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In the past several decades, numerous reports have indicated that Harmful Algal Bloom (HABs) activity is increasing on a world-wide basis. In order to test this hypothesis, we have examined 40 years of Paralytic Shellfish Poisoning (PSP) data collected by the Washington Department of Health. Due to the volume of data, we concentrated our efforts on data for Puget Sound. We have chosen to analyze these data by selecting the highest annual toxin levels in all shellfish species collected to calculate the decadal average at each site. Decadal average is defined as the average of the highest annual PSP level over a ten year period. Despite the fact that the available dataset is limited, both in sampling frequency and location of sample collection, we noted an increasing overall trend of PSP levels in this dataset, especially in the Southern portion of Puget Sound. We found that in recent years levels of PSP appear to have risen at a total of about a dozen sampling sites in the Puget Sound basin. In addition, areas within the Sound, which have been 'historically' unaffected, are now experiencing intense outbreaks of PSP. We also observe a strong correlation between population growth and PSP activity in the Puget Sound basins. Nevertheless, there are significant limitations that must be considered in extending conclusions from this dataset and these will be discussed. Recommendations for future collections and additions to the dataset will also be suggested.

PSP-LIKE TOXINS PRODUCTION BY DIFFERENT PARTICLE SIZE-FRACTIONS IN THE ST. LAWRENCE ESTUARY, CANADA

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Paralytic Shellfish Poisoning (PSP) is a recurrent phenomenon in the St. Lawrence estuary, eastern Canada, frequently leading to the closure of shellfish harvesting areas. In the Estuary, PSP outbreaks are usually well correlated with blooms of *Alexandrium tamarense*. However, a preliminary study (Levasseur *et al.*, 1996) has suggested that bacteria could also produce PSP-like toxins in the St. Lawrence estuary. The goal of the present study was to further explore the capacity of bacteria to produce PSP-like toxins and to evaluate the potential influence of this production on shellfish toxicity. From June to September 1995, over 100 L of surface seawater was collected weekly at one coastal station in the Lower Estuary and size-fractionated in three main size classes of particles: 0.22-5, 5-15, and >15 μm. Each class of particles was investigated for its content of 'PSP-like' toxins, as determined by High Performance Liquid Chromatography (HPLC). The potential to produce PSP-like toxins by those particles was determined by incubating (24h, in the dark) the material collected on the different filters. The concentration of *Alexandrium* spp. cells in each seawater sample was determined, and the toxicity of the blue mussel *Mytilus edulis* collected at the same sampling station, determined by mouse bioassay and HPLC. PSP-like toxins were found in all classes of particles measured, with the highest concentration in the >15 μm size-fractions. Results from the dark incubation show that particles from all size classes can produce PSP-like toxins. This production of PSP-like toxins can be attributed to free bacteria (0.22-5 μm) or attached bacteria (5-15, and >15 μm). However, in 1995, the contribution of bacteria to the total toxin burden was negligible and could not contribute to the intoxication of the shellfish.

HPLC WITH ELECTROCHEMICAL OXIDATION FOR THE ANALYSIS OF PSP TOXINS - IS IT TIME FOR ANOTHER INTERLABORATORY TRIAL?

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High performance liquid chromatography (HPLC) is a powerful tool for the analysis of PSP toxins such as saxitoxin found in shellfish. Saxitoxin does not absorb at wavelengths greater than 220 nm, so the PSP toxins are first oxidized to form a fluorescent derivative for detection. This oxidation can be done either chemically (i.e. HPLC-PRCS) or electrochemically (HPLC-ECOS). Both methods show excellent correlation with the results obtained using the mouse bioassay for PSP toxicity. In scallops (*Placopectin magellanicus*), the R-squared values with n = 40 were 0.94 (HPLC-PCRS) and 0.92 (HPLC-ECOS). Similar results have been observed for geoduck clams (*Panopea generosa*) where the R-squared values for n = 25 were 0.99 (HPLC-PCRS) and 0.96 (HPLC-ECOS). Despite this close agreement and its widespread use in a research setting, HPLC with either electrochemical or chemical oxidation has not received widespread acceptance for PSP analysis in a monitoring setting. This is, in part, due to the difficulties in setting up and operating the HPLC-PCRS system. These difficulties were evident in the recent interlaboratory trials for AOAC approval of the PCRS system where multiple laboratories showed widespread divergence in the values obtained for standard mixtures. HPLC-ECOS offers both advantages and disadvantages over the PCRS system. The electrochemical cell is easier to operate and more stable than the chemical oxidation, but it is also strongly affected by sample preparation and different shellfish types. Interlaboratory trials such as the one done for the PCRS approach are expensive and should only be done if there is a clear need to the technique. This poster will be to discuss the set-up and relative advantages inherent in the HPLC-ECOS approach for PSP toxins, with the goal of identifying laboratories interested in setting up ECOS systems and participating in such interlaboratory trials. (Work supported by New York Sea Grant).

ANALYSIS OF PSP TOXINS BY LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY

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Paralytic shellfish poisoning (PSP) toxins are potent neurotoxins produced by several marine dinoflagellates. The accumulation of these toxins in wild and cultured shellfish during "red tide" episodes can have serious economic and public health repercussions in affected coastal regions. Freshwater cyanobacteria are also known to produce the same toxins and deaths of wildlife and domestic animals following the ingestion of contaminated water have been widely reported. PSP toxins are tetrahydropurine derivatives based on the parent compound, saxitoxin (STX), and can be divided into three classes: carbamate, N-sulfocarbamoyl and decarbamoyl toxins. Over 20 structural analogs have been identified. The toxicity of STX is attributed to the reversible blockage of voltage-activated sodium channels on excitable cells. The various PSP toxins have different toxicities, with the carbamate toxins being the most toxic and the N-sulfocarbamoyl toxins being the least toxic. The great variety of closely related toxin structures and the varying toxicities present significant challenges to the analytical chemist interested in developing a method for their detection and quantitation. Functional assays such as the standard mouse assay, cytotoxicity assays, and receptor assays are very useful for measuring the overall toxicity of a sample, while immunoassay methods offer the potential for very rapid and inexpensive screening of samples. However, none of these assay methods provide detailed information on the toxin profile in samples. The most widely used analysis method is ion-pair LC using reversed phase columns coupled with post-column oxidation and fluorescence detection. Typically the method requires three separate runs for the analysis all the toxins. In this paper we will present a new analysis method that allows the determination of all PSP toxins in one single run. It is based on the use of hydrophilic interaction liquid chromatography (HILIC) coupled with electrospray ionization tandem mass spectrometry (MS/MS) detection. Application to real plankton and shellfish samples will be demonstrated.

PURIFICATION, CHEMICAL CHARACTERIZATION AND STABILITY STUDIES OF PARALYTIC SHELLFISH C2 TOXIN PRODUCED FROM *ALEXANDRIUM TAMARENSE*

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Our laboratory has been engaged in mass production of paralytic shellfish toxins (PST) produced by a local strain of the dinoflagellate *Alexandrium tamarense* ATCI01, which produces C2 toxin (C2T) as a predominant member of PST. In the present study, purification, chemical characterization and stability study of PST extracted from algal cells were described. Tens of milligrams of highly purified C2T and gonyautoxin 3 (GTX3) were obtained by passing the crude toxin extracts through a series of gel filtration column, reversed phase column and again gel filtration column. Purity and chemical identity of the toxins were determined by high performance liquid chromatography with fluorescence detection (HPLC-FLD), thin layer chromatography (TLC), ^{1}H and ^{13}C NMR spectroscopy and electrospray ionization mass spectra (ESI-MS). C2T was quantified by ^{1}H NMR spectroscopy using *tert*-butanol as an internal standard and by converting to GTX3 that was measured by HPLC-FLD. C2T was relatively stable at pH 3-5 and partially converted to C1 toxin at pH 6-10. C2T was hydrolyzed to GTX3 in HCl and degraded to non-fluorescence detectable metabolite in NaOH.

HETEROSIGMA AKASHIWO BLOOMS: WARNING SIGNS FOR CAGE SITES

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ABSTRACT

Experience with harmful blooms of *Heterosigma akashiwo* at the Pacific Biological Station's experimental fishfarm facility suggests that 2 indicators may give useful forewarning of impending *Heterosigma* blooms. Long-term, depression of mean monthly salinity by 1-3 ppt from multi-year means for late spring months may indicate increased risk of bloom in summer. Short-term, marked and rapid increase in water clarity may indicate that a bloom event is building to a peak within 1-2 d following.

INTRODUCTION

Like many fish culture operations the world over, the Experimental Mariculture Facility of the Pacific Biological Station at Nanaimo BC has experienced blooms of plankton species causing mortality in captive fish stocks. We raise mostly salmonids, Atlantic salmon as well as Pacific species. Our facility opened in 1974, and initially we lost fish stock to the diatom *Chaetoceros convolutus* (Kreiberg, 1988; Kennedy *et al.*, 1993). However, since 1989 we observed a shift to *Heterosigma akashiwo* as the main causative organism. Early instances of *Heterosigma* blooms caused only minor losses, 2.2% of all stocks in 1989, and 1.1% in 1991. But deadly blooms followed these in the summers of 1993 and 1997, with overall stock losses of 78% in 1993 and 75% in 1997. Examination of our records of mortality, *Heterosigma* abundance and environmental conditions suggested that two factors may provide useful advance warning of blooms to fish culturalists at cage sites.

MATERIALS AND METHODS

Samples for *Heterosigma* determinations were taken at mid-d, 0.5 m below sea surface from a single reference point on the facility. Samples of 1-10 mL were fixed with Lugol's fixative and allowed to settle for 1-5 hrs depending on abundance, subsampled if necessary and counted on a Wild M40 inverted microscope at 100X magnification and extrapolated to cell numbers per liter of seawater. Water clarity readings (Secchi disc visibility limit) and salinity samples were collected from the same reference point, the latter being taken at depths of 1 m and 4 m and measured with a refractometer.

RESULTS AND DISCUSSION

Long-term warning: we observed that in both of the years where major *Heterosigma* blooms occurred, there appeared to be a springtime priming action of reduced mean salinity (from daily observations pooled for 1-m and 4-m depth). Both bloom years saw a drop in mean monthly salinity of 1.5 to 3 ppt compared to the long-term averages for three months during the late spring (April, May and June)(Fig. 1). In non-bloom years, the monthly salinity means generally remained much nearer or slightly above the long-term monthly mean during this "early warning" window (Fig. 2). Our farmsite is within reach of the influence of runoff water from the Fraser River, a major freshwater drainage basin. Yamochi and Abe (1984) suggested that episodes of high runoff are a possible contributing factor to the observed incidence of *Heterosigma* blooms.

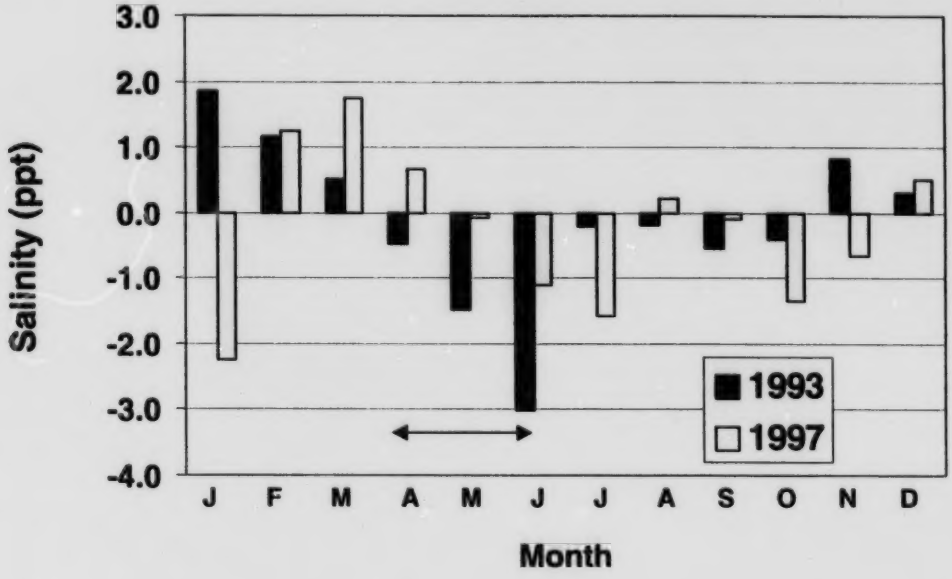

Fig. 1. Monthly mean salinity in the 2 bloom years relative to multi-year mean salinity for each month (the zero-line). The early-warning window is shown by the arrow-bar. Contrast this data with Fig. 2.

Short-term warning: *Heterosigma* blooms at our site to date were not a part of general blooms of other planktonic organisms common to the area. Honjo (1992) reported that *Heterosigma* blooms markedly reduced numbers of some other plankters via compounds produced during their growth. In our experience, *Heterosigma* blooms were often preceded by a substantial increase in water clarity a day or two prior to large increases in cell numbers (Fig. 3). We attribute the change in clarity to a broad reduction in numbers of other planktonic organisms while *Heterosigma* is increasing. This effect was seen to occur in four of the six lethal blooms experienced to date as well as on one of the three occasions where cell counts spiked above 5 million cells per litre without causing mortalities in salmon stocks. The mean Secchi readings for the nine spikes with counts in excess of 5 million cells per litre show a 50% increase in water clarity from four days before a bloom to two days before a bloom.

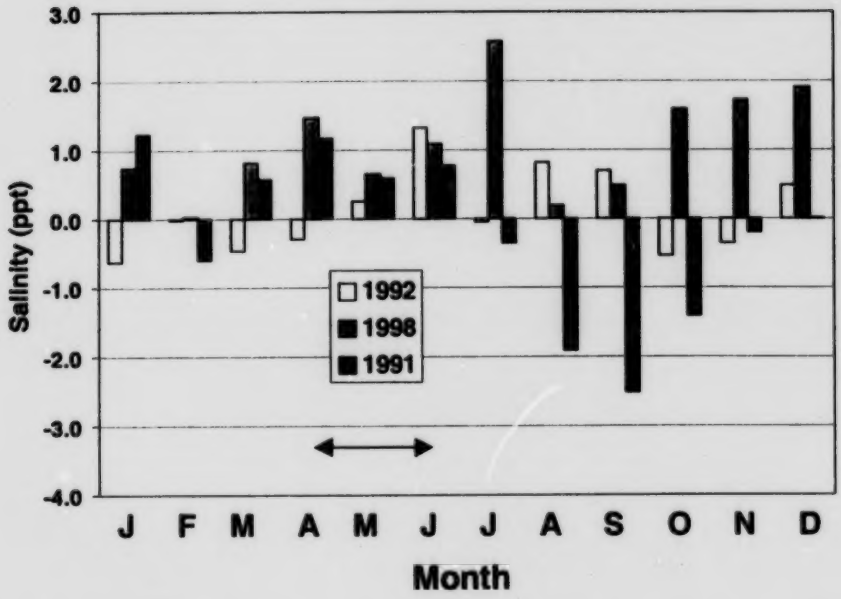

Fig. 2. Monthly mean salinity in some typical non-bloom years, relative to the multi-year monthly mean (the zero-line). Salinity during the early-warning window (shown again by the arrow-bar) is often slightly above average, as bloom years tend to depress the multi-year mean in this period.

CONCLUSION

Our experience with *Heterosigma* blooms over a period of about ten years suggests that 1) there is increased risk of a summer bloom if monthly salinity means are depressed by 1 - 3 ppt relative to long-term monthly means during late spring, and 2) a rapid and large increase in water clarity may signal that a *Heterosigma* spike is likely to occur within 1-2 d.

Plankton blooms in general appear to be driven by a range of factors, and we do not suggest that the warning signs we report here are infallible. Indeed, we have observed lethal *Heterosigma* blooms on occasions where no short-term increase in water clarity preceded them, and some heavy blooms were not toxic to our fish stocks. Neither is it established that the factors we report here are directly or causally linked with blooms. Nonetheless, our observations appear to provide 2 predictive tools which, given the difficulty of managing bloom hazards in marine animal culture, may be of practical value both to the commercial operator and the student of phytoplankton blooms.

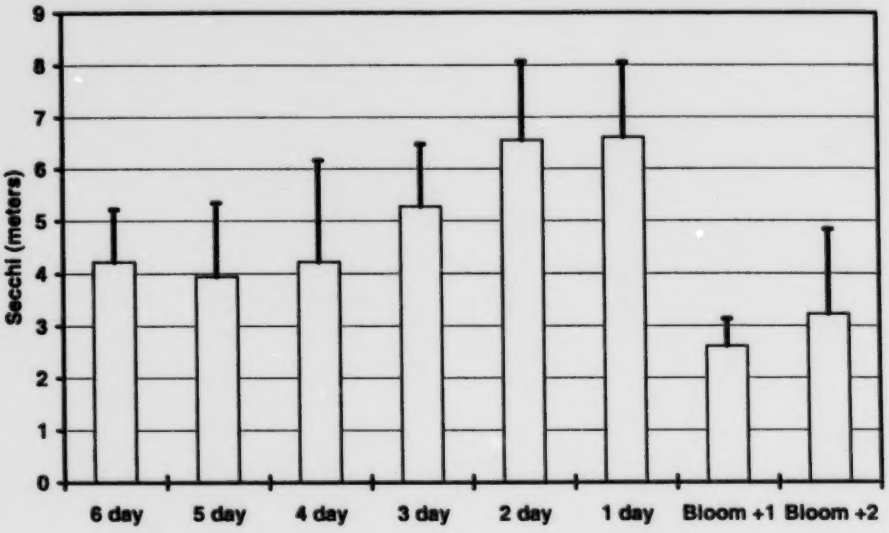

Fig. 3. Secchi disk visibility changes leading up to and during a sharp spike in *Heterosigma* cell counts (data from 5 separate incidents). Mean ± 2 SE.

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IDENTIFICATION OF THE MARINE FLAGELLATE *HETEROSIGMA AKASHIWO* IN THE SEDIMENTS OFF THE COAST OF BRITISH COLUMBIA

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High concentrations of *Heterosigma akashiwo* have been recorded for several areas of British Columbia, including Desolation Sound, Sechelt Inlet, and Sansum Narrows. In several of these areas where fish farming operations have been established there have been reports of fish kills directly associated with blooms of *H. akashiwo*. At this point in time we are not certain if the increased reporting of *H. akashiwo* is due to an increased frequency of the alga in the coastal waters or an increased awareness and recognition (via increased fish farming activities). In order to ascertain the spatial and temporal record of *H. akashiwo* we have initiated a study of its distribution in sediment cores collected from a variety of coastal locations in the Strait of Georgia, BC. *H. akashiwo* blooms could be recorded in the sediments through deposition of cysts, cells or debris. We have focused on examining for the presence of intact cysts as well as the recognition of lysed cells through the use on polyclonal antibodies and remnant pigment signatures (determined through HPLC analysis). We have also considered parameters in the core composition that would indicate other localized upper water activities. These include the overall productivity (organic composition), major species (diatom frustule analysis), and watershed changes (pollen analysis). Through our analysis we hope to recreate the historical record of the harmful algal species.

SEASONAL VARIATION IN THE ABUNDANCE OF VIRUSES INFECTING *HETEROSIGMA AKASHIWO* ON THE BC COAST

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Heterosigma akashiwo is a toxic bloom-forming alga responsible for extensive mortality of wild and farmed finfish around the world. Viruses causing lysis of *H. akashiwo* have been detected in the sediments and water column of the Strait of Georgia and adjacent inlets. Their presence implies viruses are involved in the mortality of this alga. Determining the seasonal changes in the abundances of these viruses is essential for assessing their role in *H. akashiwo* mortality. To address this question, 40 L seawater samples were collected weekly from May to September 2000 and bi-weekly or monthly from September 2000 to March 2001 in English Bay, Vancouver, BC. These water samples were 0.45 μm filtered, and the particulate material in the <0.45 μm size fraction was concentrated 200 to 250 fold using a 30,000 MW cut-off ultrafiltration cartridge. The presence of lytic agents in the concentrate was determined by infecting *H. akashiwo* NEPCC 522 cultures with 1 L seawater equivalents (approximately 5 mL) of the concentrate. The detection limit for viruses was 1 L^{-1}. In positive samples, the concentration of lytic agents smaller than 0.45 μm was determined using a most-probable-number (MPN) assay. Lytic agents were detected in all viral concentrates from May 19th to August 19th 2000 with the exception of one collected July 6th 2000 and their abundance ranged from 10 and 17,000 L^{-1}. Since these agents were filtered through a 0.45 μm filter, these agents are most likely viruses. During the period when viruses were detected, the salinity ranged from 9 to 19 PSU and the surface water temperature between 14 and 19 °C. These conditions are consistent with those that are optimal for the growth *H. akashiwo*. The presence of lytic agents in the smaller than 0.45 μm size fraction during the spring and summer suggests that viruses are important mortality agents of *H. akashiwo* in nature.

EXTRACELLULAR COMPOUNDS THAT INHIBIT CA^{2+}-ATPase TRANSPORTERS AS POSSIBLE TOXINS FROM THE ICHTHYOTOXIC ALGA *HETEROSIGMA AKASHIWO*

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Toxin(s) from the ichthyotoxic alga *Heterosigma akashiwo* have been responsible for millions of dollars of lost aquaculture stocks around the globe. Proposed mechanisms of toxicity include the production of reactive oxygen species (ROS), the release of large quantities of mucus from mucocysts, and/or the production of an organic toxin that may be brevetoxin-like in structure. However, to date, none of these postulates has been confirmed and recent evidence suggests that an alternate proposal is needed. We have taken unique steps in order to collect and test the bioactivity of extracellular organics from *H. akashiwo* cultures on cytosolic free calcium levels ($[Ca^{2+}_i]$) in the model Sf9 insect cell line. Calcium is a signaling second messenger in all organisms that controls many facets of cellular activity, including programmed cell death (apoptosis). In real-time fluorometric monitoring of $[Ca^{2+}_i]$, exposure of the organics elevated $[Ca^{2+}_i]$ in a concentration-dependent manner quickly and transiently up to 125 nM above basal (2-fold elevation). Cytosolic Ca^{2+} stimulation initially peaks, followed by a sustained plateau that is maintained for the duration of the experiment (> 15 min). Furthermore, injection of carbachol (receptor-activated Ca^{2+} release from intracellular stores) or thapsigargin (inhibits cytosolic Ca^{2+} uptake by inhibiting organelle Ca^{2+}-ATPase transporters) prior to injection of the *Heterosigma* organics potentiates the organic response by inducing an elevation of >250 nM of $[Ca^{2+}_i]$. However, the response observed by the addition of the organics is dependent on the presence of extracellular calcium, as observed by a lack of a Ca^{2+}_i response when the cells are suspended in Ca^{2+}-free buffer. Collectively, our data indicate that the unknown compound is inhibiting the plasma membrane Ca^{2+}-ATPase transporter.

PRELIMINARY RESULTS FROM PHYTOPLANKTON AND ENVIRONMENTAL MONITORING, WESTERN WASHINGTON, USA

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Washington State has a long history of harmful algal blooms as indicated by the presence of toxin in shellfish and mortalities of pen-reared salmonids. However, little is known about the temporal and spatial distribution of the causative algal species. Consequently, selected sites on the Washington Pacific Ocean coast and on inland waters of Puget Sound were monitored with varying frequency since 1990. Emphasis was on determining the presence and distribution of potentially harmful species: *Pseudo-nitzschia* spp., *Alexandrium* spp., *Dinophysis* spp., *Chaetoceros* spp., and *Heterosigma akashiwo*. Here we present preliminary results from five coast and five Puget Sound sites that were sampled twice monthly (weather permitting) from May 1997-January 2000. Water was collected with buckets while wading in shallow water at the ocean beaches and poured through a 20 μm mesh net to concentrate cells; at the inland sites cells were collected directly with a net, and surface water was dipped with a bucket at local piers. Live phytoplankton samples were analyzed using light microscopy and selected preserved samples were examined with scanning electron microscopy. Potentially harmful species were present at one or more sites in all months except March 1999. Further, all potentially harmful genera occurred at all sites except that *Alexandrium* was not positively identified in Hood Canal. *Alexandrium* was found sporadically and never in high numbers at open coast beaches. In the fall of 1997, it was present at Allyn in southern Puget Sound when record high levels (to nearly 7,000 μg/100 g) of PSP toxin were reported by the Washington Department of Health. *Pseudo-nitzschia* spp. were common on the open coast with blooms at Grayland in July 1997 (no domoic acid reported) and at Kalaloch in September 1998 when record high levels of domoic acid (287 μg/g) occurred in razor clams. *Pseudo-nitzschia* spp. were also common at Puget Sound and Hood Canal sites, but rarely occurring in high concentrations. *Dinophysis* spp. were present primarily in spring to summer and at all sites. *Heterosigma akashiwo* was rarely present, but a small bloom occurred at Allyn in October 1999. Environmental conditions included synchronous patterns of temperature at the five ocean beaches. Most of the time they were within 3 °C of each other. Inland, however, the main basin of Puget Sound (Manchester) remained cooler than either South Sound (Allyn) or Hood Canal (Twanoh, Hoodsport, Pt. Whitney) in the summer. Manchester also experienced minimal fluctuations in salinity compared to any of the other sites, which were more influenced by fresh water influxes from nearby rivers or increased salinity during coastal upwelling. There were extended periods of < 0.5 μM nitrate on the ocean beaches with episodic replenishment due to upwelling events or seasonal changes in the coastal currents. The Puget Sound main basin seldom had < 5 μM nitrate due to strong tidal mixing and weak stratification; in contrast, Hood Canal and southern Puget Sound generally had < 0.5 μM nitrate concentrations throughout the summer periods.

NORTH COAST WATER QUALITY AND BIOTOXIN PROGRAM

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The North Coast has not had a water quality and biotoxin testing program since the federal government ceased monitoring biotoxins in this area in 1964. The absence of such a program is a serious barrier to aquaculture and new fisheries development, as access to a testing program is a requirement for an aquaculture license. The lack of biotoxin monitoring also puts the health and safety of residents and visitors at risk. Furthermore, although First Nations residents are guaranteed access to shellfish for food, social and ceremonial purposes under Section 35 of the Constitution, they are officially prohibited by Fisheries and Oceans Canada from harvesting shellfish on the North Coast due to the lack of biotoxin monitoring.

The North Coast Water Quality and Biotoxin Program started out as a community driven pilot project that was initiated in 1998 and expanded in 1999. The goal was to re-establish an official biotoxin sampling and testing program for the North Coast of British Columbia. Participants include two lease sites (Humpback Bay and Kagan Bay) and the First Nations communities of Metlakatla, Kitkatla, Kitimaat, Lax Kw'alaams, Hartley Bay, and Haida Gwaii.
Biotoxin and phytoplankton sampling occur weekly from May 1- October 31st and bi-weekly from November 1- April 30th. Currently there are 10 biotoxin monitoring stations; six of these are also phytoplankton monitoring sites. Up to 17 more biotoxin monitoring stations may be established in the near future. Three biotoxins are monitored: Paralytic Shellfish Poisoning, Amnesiac Shellfish Poisoning and Diarrhetic Shellfish Poisoning.

Northern Laboratories Ltd., based in Prince Rupert, is the only lab in Canada which has been approved to both: 1) prepare PSP and ASP extracts for the Canadian Food Inspection Agency, and 2) process fecal coliform tests for Environment Canada. Biotoxin samples must reach the lab within 24 h of collection, and water samples must reach the lab within 6 h of collection. Given the remote location of most of the North Coast communities participating in the North Coast Water Quality & Biotoxin Program, this local laboratory capacity is vital.
Several alternative technologies have been investigated since the inception of this program in a series of studies. These include the MIST Quanti test, the MIST ALERT test, phytoplankton monitoring and the use of satellite data as a predictive tool for toxic algal blooms.

Currently Dr. Barb Shaw, Ocean Ecology, analyzes regular phytoplankton samples and provides reports on the prevalence of *Alexandrium*, *Pseudo-nitzschia* and *Dinophysis* species at each of the six monitoring stations. The *Alexandrium* and *Pseudo-nitzschia* results are correlated to the mouse bioassay results for PSP and ASP. In June 2001, Environment Canada will be conducting shoreline surveys and fecal coliform testing in Hartley Bay, Kitimaat, Kitkatla, Humpback Bay, Oona River, Metlakatla and Lax Kw'alaams. Survey results will be used to classify these growing areas (approved, conditionally approved or closed) at the next meeting of

the Pacific Shellfish Classification Committee on October 10, 2001. This means that by fall 2001, both Environment Canada and the Canadian Food Inspection Agency's testing requirements will have been completely fulfilled. Areas of the North Coast will be ready to be opened pending approval by Fisheries and Oceans Canada.

This program is supported by: Community Futures Development Corp., the Community Economic Adjustment Initiative, Prince Rupert Economic Development Commission, Fisheries Renewal BC, the Skeena Queen Charlotte Regional District, Northern Laboratories Ltd., the Haisla Fisheries Commission, and the Canadian Food Inspection Agency.

GYMNODINIUM CATENATUM IN NEW ZEALAND: A NEW CHALLENGE FOR PUBLIC HEALTH AND THE SHELLFISH INDUSTRY

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Between May 2000 and February 2001, a bloom of the toxic dinoflagellate *Gymnodinium catenatum*, a species previously not known in New Zealand waters, led to the widespread contamination of shellfish with PSP toxins around the coast of the North Island. This event was the most extensive, and potentially one of the most harmful, toxic algae blooms to be documented in New Zealand to date. There were few accounts of human illness associated with the event but the bloom led to health authorities issuing warnings to the public to refrain from collecting shellfish from over 1,500 km of coastline, for periods of up to at over nine months in some regions. Although the main commercial shellfish growing areas were only marginally affected, the bloom had a major impact on the shellfish industry throughout the country. This was due to prohibitions being placed on the movement of juvenile shellfish (mussels and oysters) from affected to non-affected areas, because of the risk of translocating *G. catenatum* resting cysts. Unfortunately, much of the premium seed source for the oyster and mussel industries lay within affected areas and as a result future production was threatened. Remedial measures were eventually successful in overcoming this problem. Time will tell whether the now widespread distribution of resting cysts around the coast will lead to the generation of new blooms and a possible expansion in the range of this species.

The development and progression of the bloom

Contamination of mussels with low levels of PSP-toxins (35 μg STX equivalents/100g) were first detected by the NZ Ministry of Health's marine biotoxin monitoring programme, in samples collected on 15th May 2000 from the Manukau Harbour on the north-west coast of the North Island (Fig. 1). The subsequent northward and southward progression of the bloom from this area was reflected in the waxing and waning of PSP-toxin levels around the North Island (Fig. 2). By mid November the bloom had spread around the south coast of the North Island and up the east coast as far as Hawke Bay. The highest level of PSP toxicity recorded was 4,027 μg STX equivalents/100g in Greenshell mussels from Waimamaku in Northland. Of all the positive bio-assays attributable to *G. catenatum* contamination (a total of 537 up to 9 Feb 2001), 17.5% and 7.3% were at levels >500 and >1000 μg STX equivalents/100g, respectively.

From mid September to early December, *G. catenatum* cells were often observed in Cook Strait and around the offshore margins of the Marlborough Sounds though fortunately it did not penetrate the main mussel farming areas within Pelorus Sound. Low numbers (500 cells/L) of motile *G. catenatum* cells and numerous resting cysts where found in

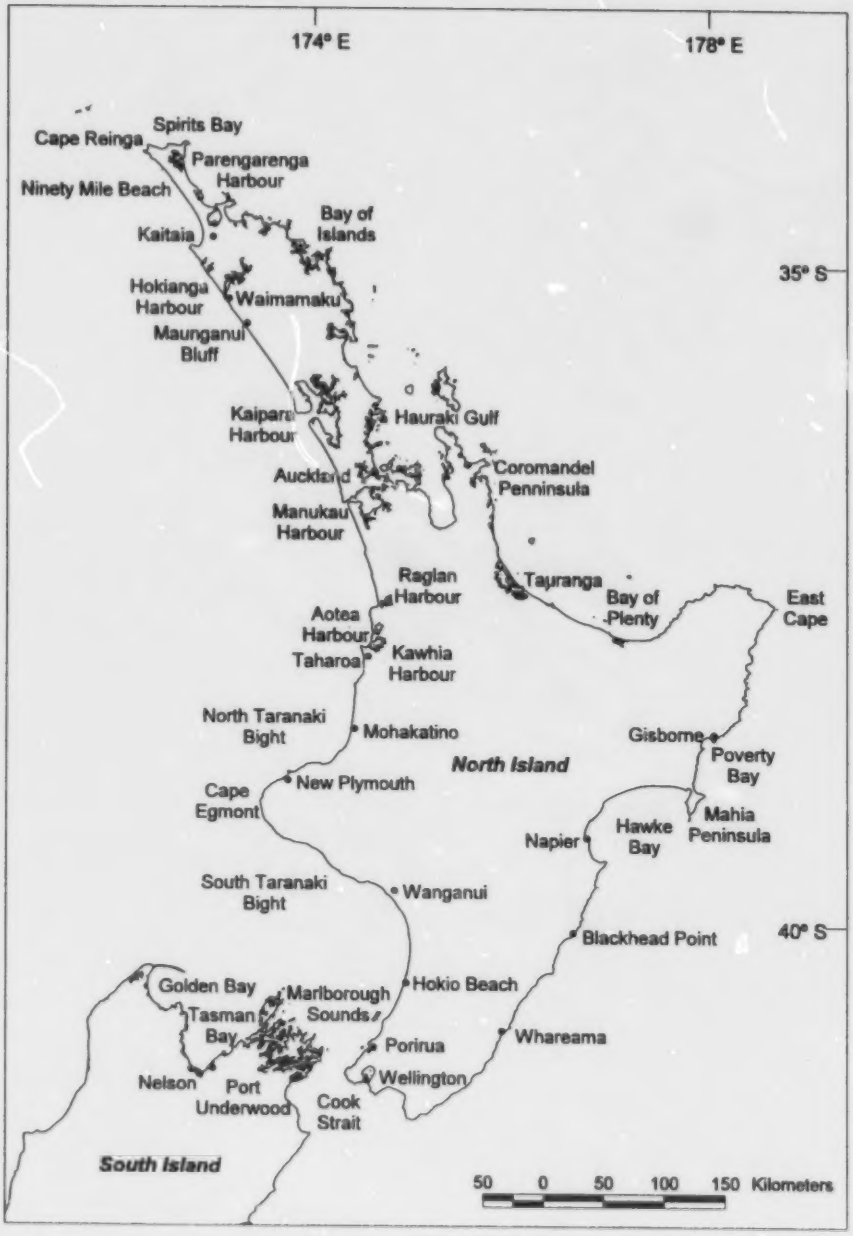

Fig. 1. Geographic locations referred to in the text.

oyster cages and on mussel ropes in Port Underwood and Tory Channel in late September, prompting the industry to place a ban on the movement of spat or mature shellfish from these areas.

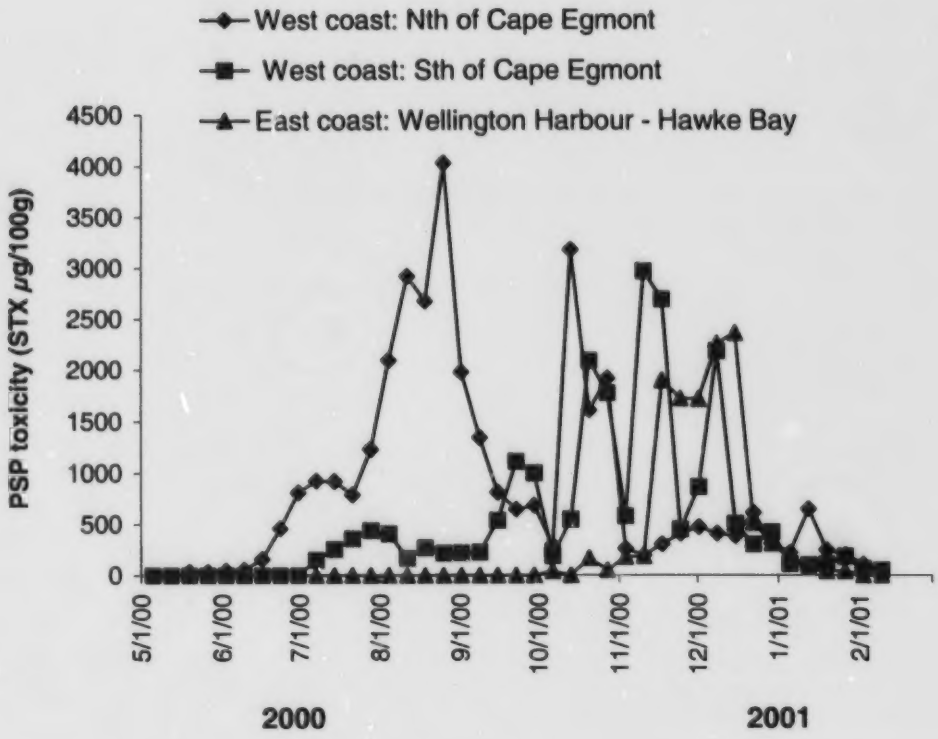

Fig. 2. North Island regional maximum PSP-toxicity scores in shellfish (various species) contaminated with *Gymnodinium catenatum* toxins, May 2000 to January 2001.

Cell and cyst morphology

The morphology of motile cells and resting cysts (hypnozygotes) of New Zealand *Gymnodinium catenatum* isolates are identical to descriptions (e.g. Blackburn *et al.*, 1989) of the same species from elsewhere in the world. In culture and in nature the motile form exists as solitary cells or more usually in chains of variable length up to 64 cells/chain. Analysis of the D1-D2 LSU rDNA sequences in New Zealand *G. catenatum* strains have shown these to be identical to those of all other isolates of *G. catenatum* from around the world (Miguel de Salas and Chris Bolch pers. comm.). The resting cyst (hypnozygote) of *G. catenatum* is 45-50 µm in diameter. It has a characteristic, red, pigment accumulation granule within a globular cytoplasm and a cell wall with a distinctive micro-reticulate pattern. These characteristics mean the cysts are easily distinguished from other dinoflagellate cysts in natural samples but simply on the basis of their appearance it is difficult to be certain of their viability.

Toxin profiles in NZ *G. catenatum*

Cultured cells of NZ *G. catenatum* isolates mainly produced toxin analogues eluting within the N-sulfocarbamoyl group. C1 and C2 were important components and there was a major peak

eluting after C3 which did not correspond to any known analogue. Within *G. catenatum* cells there was very little evidence of any of the toxins within the GTX and STX groups except for some minor traces of GTX5 and GTX1. The toxin profiles in all isolates started from vegetative cells or resting cysts from various locations were essentially identical

Toxin profiles in shellfish contaminated by *G. catenatum*

C toxin profiles in mussels extracted with 0.1N HCl (pH 3.5) showed that C1 and C2 were the predominant peaks with two additional peaks which did not correspond to any analogue for which standards were available. The first and most prominent of these unidentified peaks was the same unidentified compound which was dominant in the cell C-toxin profiles. The difference in the abundance of this compound relative to the abundance of C1 and C2 in the cells and shellfish indicated that it was labile, either as a result of metabolism within the shellfish and/or as a result of the acid extraction process. GTXs, not surprisingly, were more abundant than in the cells. GTX3 and GTX2 (the desulfated products of C1 and C2) were easily identified but there was uncertainty about the identity of a variety of other peaks on the chromatograms. One of these was the major component and was probably the carbamate product of desulfation and/or hydrolysis of the major N-sulfocarbamoyl observed in the *G. catenatum* cells.

Effects on human health

It proved difficult for health agencies to accurately determine the effects of the bloom on public health as only a small number of cases meeting the case definition of PSP intoxication came to light (Beauchamp, 2000). In some areas public health warnings were probably quite effective in preventing most people from gathering and eating shellfish. In other areas such as Northland, where levels of toxicity were highest and where shellfish are an important item of the diet, these warnings may have been ignored by large sections of the community. It is suspected that there were cases of mild poisoning which were not reported, though had serious cases occurred they would almost certainly have come to the attention of the public health system. The predominance of low specific toxicity N-sulfocarbamoyl toxins in the algae and shellfish probably explains the few reports of human illness.

***Gymnodinium catenatum*: indigenous or exotic?**

Since 1994, a routine toxic phytoplankton-monitoring programme involving the weekly sampling of 60-70 sites throughout New Zealand has been in operation (Todd, 1999). *Gymnodinium catenatum* was definitely never seen in any of the tens of thousands of samples examined during this period until its appearance in the Manukau Harbour sample collected on 20 June 2000. The small amount of data that suggests an already well developed offshore bloom intruded onto the west coast of Northland (e.g. Fig. 2), argues against a near shore point source such as would be associated with introduction via shipping. In New Zealand all the major ports are on the East Coast though there are a few locations on the North Island West Coast where significant quantities of ballast water are discharged. The significance of the LSU D1-D2 rDNA sequence data is debatable and further genetic analysis (e.g. rDNA ITS sequence data) is required to address this question. The unique toxin profile in New Zealand *G. catenatum* isolates

may be evidence that the species is indigenous but has remained a part of the "hidden flora" until recently.

Effects on the fishing and aquaculture industries

The major effect of the bloom on the shellfish industry (mussels and oysters) was the denial of access to traditional sources of spat supply due to the risk of introducing *G. catenatum* into important, unaffected, production areas such as the Coromandel and Marlborough Sounds. The bloom also affected the spiny lobster (*Jasus edwardsii*) fishery when it was found that PSP toxins, with the characteristic toxin profile of *G. catenatum*, were present in the digestive gland of the animals. Although edible portions such as the flesh of the tail remained free of contamination, because most product is exported as whole live animals, a temporary, precautionary, ban was placed on this trade.

The Kaitaia spat issue and remedial measures

Prior to the bloom, the NZ mussel industry (production > 80,000 tonnes/year) obtained 80% of its seed from drift algae which is periodically cast up on Ninety Mile Beach in Northland, attached to which are very high numbers of newly settled Greenshell mussel spat. For many years this material, colloquially know as "Kaitaia spat", has provided a cheap and convenient seed source for the mussel growers. In samples collected on 23 June 2000, resting cysts of *G. catenatum* were first seen in low numbers (89 cysts/kg weed) in Kaitaia spat and the industry immediately imposed a ban on its transport and use. Subsequent examinations revealed very high numbers (>40,000/kg of weed) of *G. catenatum* cysts in some batches of Kaitaia spat and it became obvious that the ban on its use would be long term and could seriously affect future mussel production.

As a consequence of the lack of Kaitaia spat a strenuous effort was made to develop methods of separating the mussel spat from the seaweed and the dinoflagellate cysts. These efforts were successful and there are now several cleansing plants in operation, from which, in January 2001, the first cleaned mussel spat originating from Ninety Mile Beach was permitted to be placed in 'clean' growing areas of the Marlborough Sounds.

What does the future hold?

Surveys have revealed that sediments in areas where the bloom was present (i.e. large stretches of the North Island coast) contain high numbers of *G. catenatum* cysts. In May 2001, toxin residues were still present in surf clams on the Northland west coast, *G. catenatum* cysts were still present in Kaitaia spat and motile cells were observed in water samples from the outer Marlborough Sounds and Hawke Bay. Only time will tell whether the widespread distribution of resting cysts around the coast will lead to the generation of new blooms in the future and a possible expansion in the range of this species.

ACKNOWLEDGEMENTS

Thanks to Janet Young and Tony Beauchamp at the Ministry of Health for their assistance and permission to use the Ministry of Health monitoring data. Thanks also to Paul Lupi of NZ Mussel Industry Council and Helen Smale of the Marlborough Sounds Shellfish Quality Programme for permission to use data collected by the mussel industry and Janet Adamson for carrying out the HPLC analyses. This research was funded under contract CAW-12 from the NZ Foundation for Science Research and Technology. Attendance at this conference was funded by the Technical Participation Programme (TPP) of the NZ Ministry of Research, Science and Technology.

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MARINE BIOTOXIN ILLNESSES IN WASHINGTON STATE 1942 TO 2000 "WHAT YOU DON'T KNOW CAN HURT YOU"

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The Department of Health (DOH), Office of Food Safety and Shellfish Programs, is charged with protecting shellfish consumers by monitoring biotoxin levels and, when necessary, issuing recalls and health advisories concerning elevated toxin levels. To accomplish this task we depend on the widespread partnerships that we develop with Local Health Jurisdictions, Volunteers, the Commercial Industry, other State Agencies and Tribes. We test molluskan shellfish samples from both the commercial industry and public beaches. In August 2000, nine South Puget Sound recreational harvesters became seriously ill as result of consuming Blue Mussels with increased PSP levels, some above 3000 μg/100 g tissue. The mussels were harvested from both public (boat Launch) and Private (dock) property. Of the nine, four were hospitalized and two required ventilation for several days. Fortunately, with the immediate availability of emergency medical care, there were no deaths. Washington State has been conducting routine biotoxin monitoring since 1931. Since 1942, three deaths and numerous illnesses have occurred in Washington that can be attributed to Paralytic Shellfish Poisoning and/or Domoic Acid. In response to these events, DOH has increased PSP sampling from 200-300 samples per year, to 3000-3700 samples per year. To accomplish this we have developed partnerships not only for monitoring, but also to convey health advisory information, including translation to the non-English speaking populous such as our Asian-Pacific Islander communities. Our poster presentation will outline the August 2000 illness investigation, HAB history in Washington State and our current Biotoxin Monitoring Program. Linda Hanson and Frank Cox represented the Washington State Department of Health.

COMPARISON OF GLUTAMIC AND DOMOIC ACID EXCITOTOXIC RESPONSES *IN VITRO*

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ABSTRACT

Domoic acid (DA) is a glutamate (Glu)-related excitotoxic shellfish toxin which is responsible for amnesic shellfish poisoning (ASP). The present studies were initiated to examine *in vitro* the possible cellular mechanisms responsible for increased sensitivity to ingested DA compared to Glu, a common food additive. Excitotoxin-sensitive human neuronal cells were formed by retinoic acid mediated differentiation of human NT-2 teratocarcinoma cells and their responses to Glu and DA compared. As well, the effects of Glu and DA on Glu transport across the plasma membrane were examined in normal human NHA astrocytes. NT-2 cells acquired sensitivity to both excitotoxins at a threshold concentration of 50 μM after the differentiated cells had been maintained in culture for two weeks. Excitotoxin-induced degradation of cell morphology could be observed either microscopically or by staining with propidium iodide (PI). L-phenylalanine was included as a negative control and was non-toxic at all concentrations tested. Visual evidence of excitotoxicity and PI fluorescence induced by 100 μM L-Glu were inhibited by concentrations of MK801, an antagonist for the N-methyl-d-aspartate subclass of glutamate receptors, as low as 50 nM. MK801 was unable to prevent excitotoxicity induced by 100 μM DA. DA had no effect on ^{3}H-Glu uptake into NHA cells when tested at 1 μM to 1 mM concentrations. These results suggest that an increased susceptibility to DA poisoning may be due to the inability of glutamate transporters to clear DA from the synaptic cleft, thus prolonging neuronal excitation until toxicity occurs.

INTRODUCTION

Glutamate has been identified as the major stimulatory neurotransmitter in the central nervous system; however, unregulated exposure of glutamatergic neurons to either glutamate or a number of related chemicals can lead to cell death by toxic overstimulation, a process first termed "excitotoxicity" by Olney *et al.* (1971). The marine toxin domoic acid is one such glutamate analogue which has been identified as a particular health concern. Domoic acid occurs in phytoplankton blooms as a product of the diatom *Pseudo-nitzschia multiseries*, and other species of *Pseudo-nitzschia*. Shellfish feeding during phytoplankton blooms have been found to accumulate levels of domoic acid toxic for human consumption. In Canada, consumption of mussels contaminated with domoic acid caused an outbreak of amnesic shellfish poisoning (ASP) in late 1987 which affected 107 people (for reviews, see Hynie and Todd, 1990; Todd, 1993).

Glutamate and related chemicals exert their neurotransmitter effects by interacting with specific glutamate receptors, which fall into two major mechanistic classes: 1) ionotropic receptors which gate ion channels; and 2) metabotropic receptors which are coupled to G proteins and interact with secondary messenger signalling pathways (for reviews, see Nakanishi, 1994; Nakanishi and Masu, 1994; Pin and Duvoisin, 1995; Ozawa *et al.*, 1998). Both classes of glutamate receptors have been implicated in the onset or modulation of the excitotoxic response. Ionotropic receptors have been further categorized into three subclasses based upon the pharmacological activity of various agonists and antagonists: 1) NMDA (N-methyl-d-aspartate), 2) AMPA (dl-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)/kainate and 3) high affinity kainate receptors. Several different subunit genes for each subtype of ionotropic receptor have also been identified and alternative splicing of mRNA and mRNA editing mechanisms contribute to further complexity. In addition, a similarly diverse group of genes and splice variants has been identified for metabotropic receptors. The mechanism by which glutamate receptor activation leads to excitotoxic neuronal cell death remains an area of intense investigation but is associated with calcium mobilization, inhibition of adenylate cyclase, free radical production and nitric oxide synthesis (Bondy and Lee, 1993; Coyle and Puttfarcken, 1993; Zeevalk and Nicklas, 1994; Chen *et al.*, 1995; Mody and MacDonald, 1995; Schulz *et al.*, 1995; Thomas, 1995; Xi and Ramsdell, 1996; Bhardwaj, 1997; Lancelot *et al.*, 1998; Nijjar and Nijjar, 2000).

The efficient removal of glutamate from the synapse is also critical in order to attenuate the excitatory signal before the process progresses to excitotoxicity. Astrocytes surrounding the synapse appear to perform an important role in this protective function by possessing high affinity glutamate transporters which aid in glutamate removal and modulate the excitatory response. Exposure to excitotoxins *in vivo* has been associated with glial injury (Appel *et al.*, 1997; Scallet and Ye, 1997). Increased levels of glutamate have been found in primary rat astrocytes exposed to domoic acid for 5 min *in vitro*, while a 60 min exposure was found to inhibit the accumulation of glutamate (Ross *et al.*, 2000). This effect was attributed to an inhibition of glutamate uptake mediated by domoic acid.

In this paper, the excitotoxic response of human NT-2 teratocarcinoma cells exposed to glutamate and domoate was compared, as well as effects on glutamate transport in NHA normal human astrocytes. NT-2 cells provide a unique human model for excitotoxicity studies. Cells incubated in the presence of retinoic acid are induced to terminally differentiate into non-replicating neuronal-like (hNT) cells which gain expression of mRNA for all known classes of ionotropic glutamate receptor subunits and develop sensitivity to glutamic acid and kainic acid, an excitotoxin structurally related to domoic acid (Munir *et al.*, 1995; Hardy *et al.*, 1994; Younkin *et al.*, 1993; Pleasure and Lee, 1993). NHA primary human astrocytes have been used as a human model for examining the effects of domoic acid on the intracellular transport of radiolabelled glutamate.

MATERIALS AND METHODS

Chemicals and Tissue Culture Materials

The human teratocarcinoma NT-2 cell line was purchased from Stratagene, LaJolla, CA, and NHA normal human astrocytes were purchased from Clonetics, San Diego, CA. Sterile tissue

culture plasticware was purchased from Corning, Corning, NY and Becton Dickinson, Franklin Lakes, NJ. William's medium E (WME), basal medium Eagle (BME), Hanks balanced salt solution without calcium and magnesium (HBSS), trypsin/EDTA and glutamine were obtained from Gibco, Gaithersburg, MD. Fetal bovine serum was purchased from Hyclone, Logan, UT. Gentamycin, *all trans*-retinoic acid, L-glutamic acid, L-phenylalanine, and propidium iodide were purchased from Sigma, St. Louis, MO. Cytosine arabinoside was purchased from Calbiochem, San Diego, CA. MK801 was purchased from Research Biochemicals, Natick, MA and domoic acid was purchased from Diagnostic Chemicals Ltd, Charlottetown, PEI. L-[3,4-^{3}H]-glutamic acid (41.1 Ci/mmol) was purchased from Perkin Elmer, Markham, ON.

Maintenance and Differentiation of NT-2 cells

Human teratocarcinoma NT-2 cells were maintained in 75 cm^2 tissue culture flasks containing 15 mL WME supplemented with 10% fetal bovine serum, glutamine and gentamycin. Cells were passaged by trypsinization as required. Culture conditions for differentiation of the cells into neuronal-like hNT cells were similar to those described (Pleasure *et al.*, 1992; Pleasure and Lee, 1993; Younkin *et al.*, 1993; Hardy *et al.*, 1994; Munir *et al.*, 1995) except for the substitution of WME for Dulbecco's modified Eagle's medium (DMEM). Briefly, NT-2 cells were trypsinized, resuspended at 2 x 10^6 cells/T75 flask in 15 mL supplemented WE medium containing 10^{-5} M *all trans*-retinoic acid. Incubation was continued for 5 wk, during which time medium was replaced twice each wk with fresh supplemented WME containing retinoic acid.

Selection of Neuronal Cells

The culture conditions finally chosen for selection of hNT cells were somewhat simplified compared to those described previously (Pleasure *et al.*, 1992; Pleasure and Lee, 1993). Cells were trypsinized, resuspended in supplemented BME lacking retinoic acid and cells from each 75 cm^2 flask divided into six 25 cm^2 tissue culture flasks. The hNT cells were then semi-purified by two cycles of differential harvesting as follows. After incubation at 37°C for 6-7 h, most of the conditioned medium was removed and transferred to a sterile container. The flasks were then tapped against the benchtop 10-12 repetitions per side to dislodge the neuronal cells, which attach less strongly to the plastic substrate than other cell types present in the culture. Floating cells were resuspended in 5 mL of the saved, conditioned BME, seeded into fresh 25 cm^2 tissue culture flasks and incubated overnight at 37°C in a humidified CO_2 atmosphere. The differential harvesting procedure was repeated the next morning, after which the cells were seeded into 96 well plates at 2 x 10^5 cells per well in a volume of 200 μL supplemented BME containing 20 μM cytosine arabinoside. After 7 d of incubation, the medium was removed and replaced with fresh supplemented BME without cytosine arabinoside. Incubation was continued for the indicated times without further changes of medium.

Chemical Treatment

100 mM stock solutions of L-glutamic acid, L-phenylalanine, domoic acid, and MK801 were made in HBSS. Excitotoxin solutions were adjusted with NaOH to pH 8.0, dilutions made in HBSS, and 20 μL aliquots of chemical solutions added to each well. In experiments including the glutamate receptor inhibitor MK801, inhibitor was added and the cells incubated for 30 min

before addition of glutamic or domoic acid solutions. Where necessary in control wells, HBSS was added to keep well volumes constant across the plate.

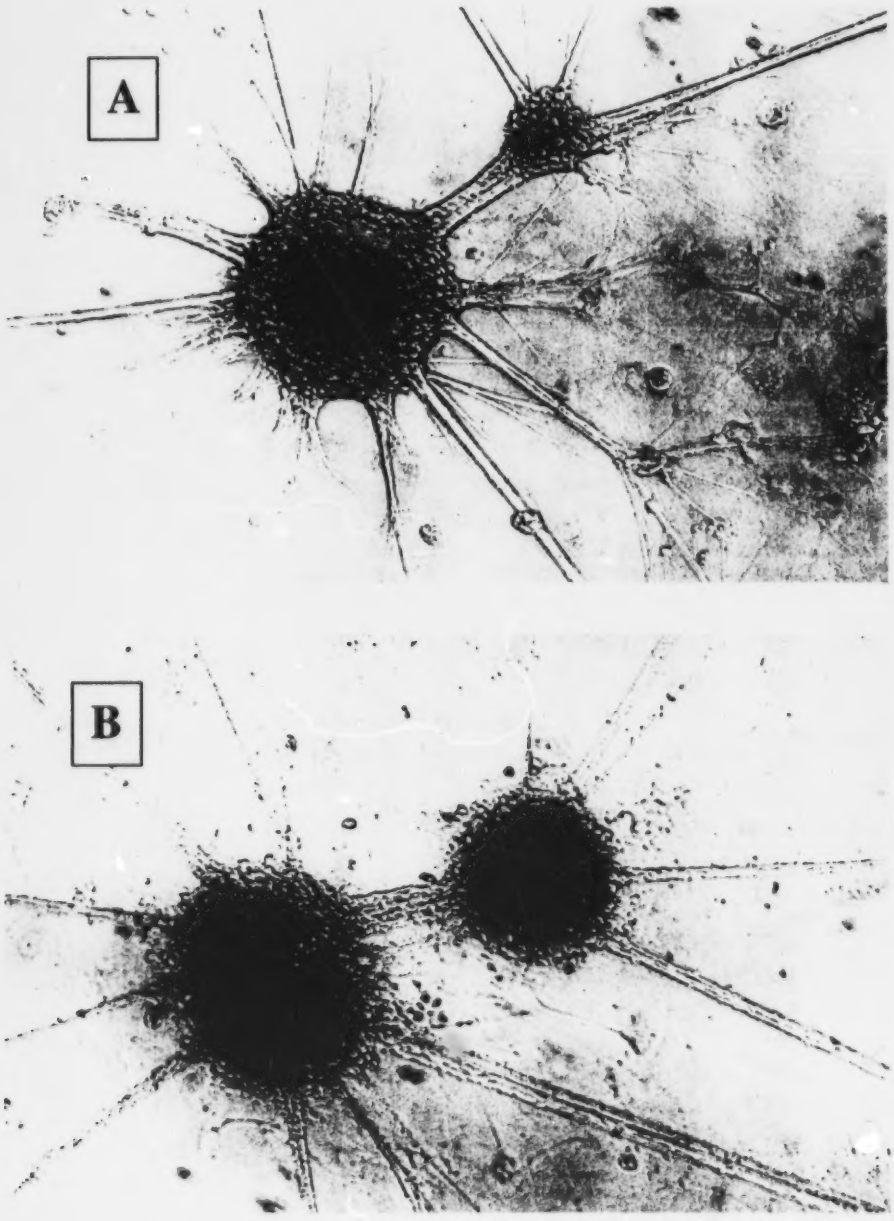

Fig. 1. Excitotoxin-induced cellular degradation. A, untreated control hNT cultures after 4 wk in culture; B, cell cultures exposed to 100 μM glutamate for 24 h.

Viability Assay with Propidium Iodide

Propidium iodide was dissolved in HBSS at a concentration of 100 μg/mL and 10 μL volumes were added to each test well. The plates were incubated at 37°C in a humidified CO_2 incubator for 45 min and the fluorescence read on a Cytofluor multiwell plate spectrofluorimeter (Millipore, Bedford, MA; excitation, 536 nm; emission, 617 nm).

NHA Cell Culture and Glutamate Transport Assay

NHA cells were maintained in Williams E medium supplemented with gentamycin, glutamine and 20% fetal bovine serum. The cells were trypsinized as needed before reaching confluence. Glutamate uptake into NHA cells was determined using a modification of the method reported by Volterra *et al.* (1994). Briefly, NHA cells were seeded at 3.3×10^4 cells per 30 mm culture dish in supplemented Williams E medium (20% FBS, L-glutamine, gentamycin) and allowed to grow to near confluency. The medium was then replaced with 1 mL of Krebs/bicarbonate buffer containing the test chemical and the cells incubated (10 min, 37°C, 5% CO_2). ^{3}H-Glutamic acid was then added (0.1 mL, 500,000 dpm, 100 μM). After either 0 or 5 min of incubation, the cells were washed and lysed with 0.2 mL NaOH. The amount of protein was determined in 0.2 mL of lysate and the level of radioactivity in the remainder of the sample was measured by scintillation counting. Glutamate uptake was then expressed as ^{3}H dpm/μg protein.

RESULTS

Changes in cell morphology occurring with exposure to excitotoxins are shown in Fig. 1. With increasing time in culture, the hNT cell bodies congregate into clusters joined by axonal-like processes. Untreated cell clusters after 4 wk of incubation (Panel A) exhibited distinct boundaries with smooth, featureless processes. Upon exposure to 100 μM L-glutamate for 30 min, swelling of the cells became noticeable (Panel B) and the sharp, well defined edges of the clusters (evident in Panel A) disappeared. The cellular degradation is progressive with increased disruption of the cells and obvious blebbing of axonal-like processes observed by 24 h of chemical exposure. These morphological changes were also observed with domoic acid.

The induction of PI fluorescence by glutamate and domoic acid in hNT cultures of increasing age is shown in Fig. 2. Excitotoxicity in differentiated hNT cells can be detected by increases in propidium iodide fluorescence; however, despite changes in cell morphology at 30 min post chemical addition, increased fluorescence becomes detectable after 4 h and increases up to 24 h. Changes in morphology as well as increases in propidium iodide fluorescence began at 50 μM concentration for both glutamic and domoic acids. Parallel cell cultures exposed to increasing concentrations of phenylalanine as control were unaffected. The effect of the NMDA glutamate receptor inhibitor MK801 on glutamate and domoic acid induced excitotoxicity is shown in Fig. 3.

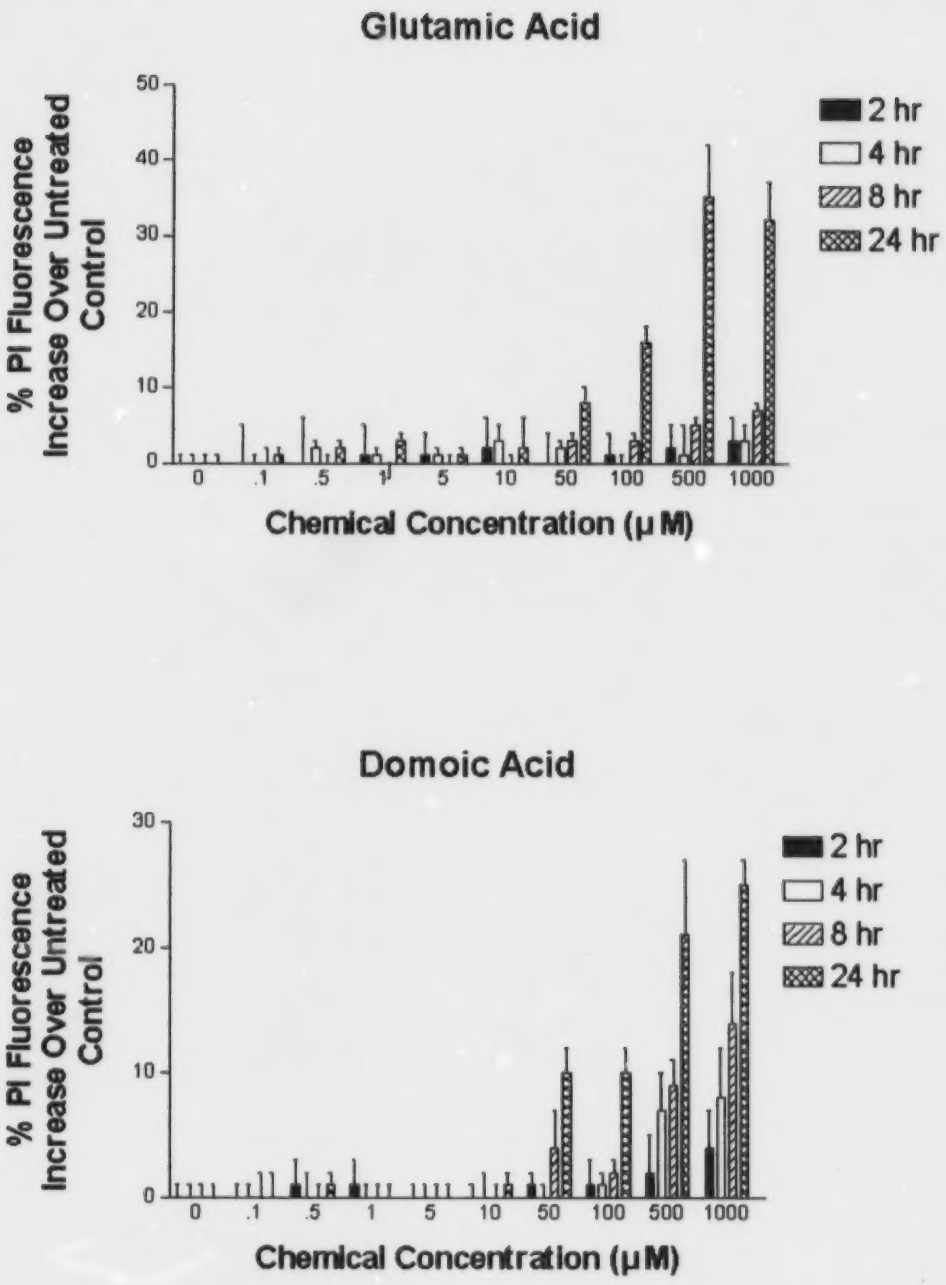

Fig. 2. Induction of propidium iodide fluorescence in hNT cells with increasing time of exposure to test chemical.

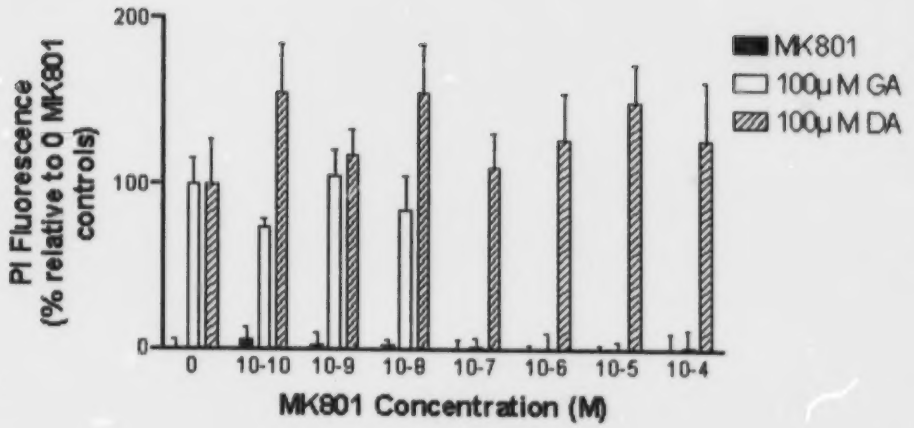

Fig. 3. Comparison of NMDA receptor antagonist MK801 inhibition of excitotoxicity in hNT cells exposed to either glutamate or domoate.

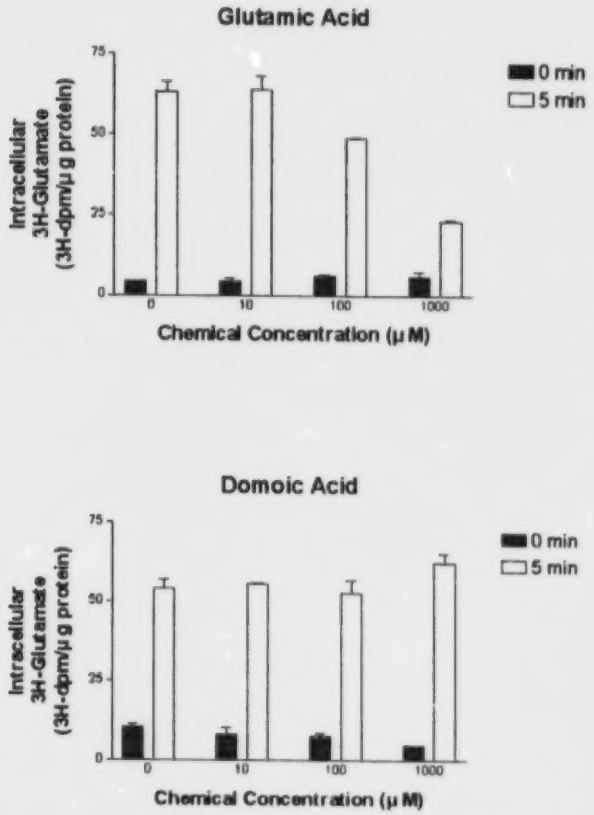

Fig. 4. Effect of glutamate and domoate on uptake of ^{3}H-glutamate into normal human NHA astrocytes.

MK801 was added to cultures 30 min prior to addition of excitotoxin; therefore, both inhibitor and excitotoxin were present over the 24 h exposure period. Concentrations of MK-801 ranging from 50 nM to 500 μM were active in protecting hNt cells against both the morphological changes and increases in PI fluorescence otherwise induced by exposure to 100 μM glutamate. At 1 mM MK801, toxicity induced by MK-801 itself became evident. MK801 was unable in inhibit the induction of PI fluorescence by 100 μM domoic acid and the cells underwent the characteristic degenerative changes in morphology associated with excitotoxicity.

The effect of increasing concentrations of glutamic and domoic acids on transport of ^{3}H-glutamate into NHA normal human astrocytes is shown in Fig. 4. Cells were preincubated for 10 min with unlabelled glutamic acid or domoic acid and then transport of ^{3}H-glutamate into NHA cells examined 5 min after the addition of radiolabelled glutamate. The addition of unlabelled glutamate led to decreased levels of radiolabel associated with the NHA cells, indicating that unlabelled glutamate was able to compete with the radiolabelled glutamate for transport into the astrocytes. No such competition was observed with domoic acid, suggesting that domoic acid is not transported into astrocytes by the same mechanism as glutamate.

DISCUSSION

Most *in vitro* approaches to the study of excitotoxicity have concentrated upon primary cultures of brain or retinal neurons, chiefly of rodent origin. Unfortunately, cytotoxic or other excitotoxin-induced effects described in neuronal cell lines have generally been characterized as atypical or non-receptor mediated (Chovanes *et al.*, 1992; Davis and Maher, 1994; Froissard and Duval, 1994; Froissard *et al.*, 1997). In the present study, the toxicities of glutamic acid and domoic acid have been compared in human neuronal hNT cells formed by retinoic acid induced differentiation of the teratocarcinoma NT-2 cell line. Characterization of glutamate receptor expression and excitotoxin sensitivity of hNT cells by others (Pleasure and Lee, 1993; Younkin *et al.*, 1993; Hardy *et al.*, 1994; Munir *et al.*, 1995), as well as the results presented in this study, demonstrate that hNT cells are sensitive to a wide range of excitotoxins. In addition, the effects of glutamate and domoate on uptake of ^{3}H-glutamic acid by normal human astrocytes (NHA cells) were compared.

The hNT cells were found to exhibit excitotoxic responses to both glutamic acid and domoic acid with excitotoxicity induced by both test chemicals at a threshold concentration of 50 μM. Morphological changes associated with excitotoxicity became evident microscopically within 20 to 30 min after addition of test chemical and the degeneration appeared to continue over a 24 h time period. Cell degeneration was also detected by means of staining with propidium iodide, with increased fluorescence generally detectable within 4 h of excitotoxin addition. The intensity of fluorescence increased over 24 h reflecting the increasing damage observed visually. Phenylalanine has not been identified as an excitotoxic amino acid and no evidence for phenylalanine excitotoxicity in hNT cells was observed up to the maximum concentration tested (1 mM). The hNT cells appear to be less sensitive to domoic acid compared to primary rat cerebellar granule cell cultures (Novelli *et al.*, 1990) which displayed an increased sensitivity to domoic acid. Whether this difference in response reflects a species or cell type specific difference in susceptibility or differences in the cell culture conditions is unknown. Factors such as pH of the test chemical and the presence of glucose in the culture medium have been shown to

affect the excitotoxic response. Excitotoxins adjusted to pH 7.4 have been found more potent than those at acidic pH (Nijjar and Madhyastha, 1997) while glucose deprivation and low cellular energy levels cause increased sensitivity to excitotoxins (Novelli *et al.*, 1988; Nijjar, 1993). In the present experiments, pH of the test chemicals was matched at pH 8 and the chemicals were applied in BME without a change to glucose free medium. The effect of glucose deprivation on hNT cells has been difficult to assess since the cells become quite fragile by the end of the differentiation and selection process, and do not tolerate medium changes. In addition, the use of propidium iodide fluorescence as a marker for viability, while very straightforward, may not be quite as sensitive as viability assays such as release of lactate dehydrogenase which has been used by others. Sub-threshold levels of glutamate or NMDA (Novelli *et al.*, 1992; Tasker and Strain, 1998) have also been shown to enhance the excitotoxic response induced by domoate, an aspect which has not been examined here.

The involvement of N-methyl-d-aspartate receptor activation in glutamate and domoate induced excitotoxicity was further examined using the NMDA receptor antagonist MK801. In agreement with previous characterizations of hNT cells (Munir *et al.*, 1995), MK801 was shown to be effective as an inhibitor of glutamate-induced excitotoxicity. However, the present studies demonstrate that MK801 was inactive against domoate-induced excitotoxicity. While activation of the NMDA receptor has been shown to be the primary mechanism involved in the onset of excitotoxicity mediated by glutamate (Novelli *et al.*, 1988), the excitotoxic response to domoic acid appears to be more complicated. Part of the excitotoxic response is initiated by the interaction of domoic acid with AMPA and high affinity kainate receptors (Tasker *et al.*, 1996; Larm *et al.*, 1997; Ferreira *et al.*, 1998; Crawford *et al.* 1999). This interaction then results in the release of glutamate and aspartate, which causes excitotoxicity through interaction with NMDA receptors (Brown and Nijjar, 1995; Malva *et al.*, 1996; Berman and Murray, 1997). However, the contribution of these different receptor subtypes to domoate excitotoxicity appears to depend to some extent upon the experimental design and particular receptor antagonists used. Glutamate and domoate elicit different responses in electrophysiological studies, with glutamate exposure resulting in rapidly desensitizing responses while domoate treatment produces non-desensitizing or slowly desensitizing responses at AMPA receptors and some kainate receptors (Hampson and Manalo, 1998). The present studies demonstrate that MK801 is capable of preventing the induction of excitotoxicity in hNT cells by glutamate but is unable to affect domoate induced excitotoxicity. These results suggest that, at least in hNT cells, domoic acid can cause excitotoxicity without a major activation of NMDA receptors and also supports previous findings that domoate exerts it excitotoxic effects through kainate ionotropic receptors (Debonnel *et al.*, 1990).

Domoic acid did not compete with radiolabelled glutamate for transport into NHA astrocytes, suggesting that the protective effect of astrocytes in removing excitotoxins from the synaptic cleft is ineffective against domoic acid. The cells were incubated with domoic acid for 10 min prior to addition of ^{3}H-glutamate and then analyzed for ^{3}H accumulation after 5 min of further incubation, a time period previously determined to fall during the linear phase of glutamate accumulation. In constrast, Ross *et al.* (2000) measured the effect of domoic acid on glutamate levels in primary rat astrocyte cultures using HPLC coupled to fluorescence detection and found elevated glutamate levels after exposure to 10 μM domoic acid for 5 min. No effect was observed with 100 μM glutamate. In the present study, no concentration dependent effect of

domoic acid on transport of ^{3}H-glutamate was observed in NHA cells incubated with concentrations of domoic acid up to 1 mM. When Ross *et al.* (2000) extended the incubation time of rat astrocytes with domoic acid to 60 min, a domoate concentration dependent decrease in glutamate accumulation was found. It was unclear whether this effect was a direct result of domoate inhibition of glutamate transport or an indirect effect on ATP production, glutamate synthesis or degradation, or other metabolic processes. The possible effect of longer term domoic acid exposure on NHA glutamate transport is currently being investigated. However, the results reported here would suggest that domoic acid does not directly compete with glutamate for transport into human astrocytes. Although the present experiments are unable to discount the existence of another mechanism for domoic acid transport, in the absence of such an alternative mechanism, domoic acid would remain in the synapse, leading to longer term neuronal stimulation and excitotoxicity. Such a mechanism may be a factor in the potent excitotoxic activity of domoic acid observed *in vivo*.

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OLYMPIC REGION HARMFUL ALGAL BLOOMS (ORHAB): WASHINGTON STATE HARMFUL ALGAL BLOOM MONITORING PROJECT

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Harmful algal blooms (HABs) are a common occurrence on the Washington coast, but our knowledge of the processes that govern their timing and spatial distributions, subsequent advection to the coast, and dispersal are not sufficient to predict the possibility of shellfish contamination in the area. In response to this need, the Olympic Region Harmful Algal Bloom (ORHAB) group was organized as a forum for collaboration and cooperation among federal, state, and local government agencies, academic institutions, coastal Native tribes, marine resource-based businesses, and public interest groups. Its mission is to support applied and basic research on HABs and to build a greater local capacity to monitor and mitigate the effects of such events. The ultimate goal is to sustain a long-term monitoring program into the future without reliance of federal support. ORHAB, a multi-agency, multi-disciplinary project funded by NOAA's Coastal Ocean Program, is investigating the origins of open-coast toxic blooms, monitoring where and when the toxic species are present on the coast, assessing the environmental conditions under which blooms occur and are transported to intertidal shellfish populations, and exploring methods that can be used to forecast HABs. The primary focus is on the presence of *Pseudo-nitzschia* spp. and domoic acid in razor clams (*Siliqua patula* Dixon). The monitoring four sites, Kalaloch, Copalis, Twin Harbors, and Long Beach, were chosen because they have harvestable razor clam populations and some also have historical phytoplankton data. The working hypothesis is that phytoplankton blooms in water over the Washington continental shelf are the source of toxins in razor clams on coastal beaches. The precise timing of physical processes (currents, winds, upwelling, and downwelling) off the coast determines whether a toxic bloom will be advected in to the nearshore region and be sustained there long enough for razor clams to become toxic. The project is determining the temporal and spatial distributions of *Pseudo-nitzschia* spp. and relating these to hydrological and meteorological parameters using standard oceanographic and biological methods. New techniques for the rapid detection of toxins and toxigenic species will be tested as they become available. The major partners/collaborators and contact persons are: Northwest Fisheries Science Center (Vera Trainer, project PI); Battelle Marine Laboratory (Dana Woodruff); Olympic Coast National Marine Sanctuary (Ed Bowlby); Pacific Shellfish Institute (Dan Cheney, Ralph Elston); Quinault Indian Nation (Joe Schumacker); Saigene Corporation (Paul Haydock); University of Washington Olympic Natural Resources Center (Miranda Wecker); University of Washington School of Oceanography (Barbara Hickey, Rita Horner); Washington Department of Ecology (Jan Newton); Washington Department of Fish and Wildlife (Dan Ayres); Washington Department of Health (Judy Dowell).

WORKING GROUP SUMMARIES

REPORT OF THE WORKING GROUP A

HAB INTERACTIONS AFFECTING FISHERIES AND POTENTIAL PROTOCOLS FOR MITIGATION OF HAB EFFECTS

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INTRODUCTION

Over the past two decades, it has become clear that one of the major effects of certain harmful algal blooms (HABs) is to cause serious economic damage to the fisheries aquaculture industry, both internationally and in Canada (Taylor and Haigh, 1993; Horner *et al.*, 1997; Whyte, 1997; Whyte *et al.*, 1999; 2001). On the West Coast of Canada, the following harmful algal species are particularly problematic: *Heterosigma akashiwo*, *Chaetoceros concavicornis*, *C. convolutus*, and recently, *Cochlodinium polykrikoides*. New potentially harmful species are also being discovered such as *Chrysochromulina hirta*?

Both the present operation and further development of finfish and shellfish aquaculture are being seriously impeded by the periodic appearance of these harmful algae. For this reason, a Working Group was convened to discuss 1) the impacts harmful algae on finfish aquaculture, and 2) the currently used and potential protocols aimed at mitigating the adverse affects of these HABs. The discussions, most of which centered around finfish aquaculture, are summarized in the following sections.

1) Impacts of harmful algae on finfish aquaculture

The discussion was focused on a specific experimental finfish aquaculture site, situated at a shallow depth (15-20 m) near DFO's Pacific Biological Station, Nanaimo, BC. Researcher Henrik Kreiberg described his experiences in with dealing with *Heterosigma* and *Chaetoceros* blooms at the research site, which has the advantage of housing a greater variety of fish species and culture histories than at commercial sites. This let him observe that the impacts vary greatly depending on the salmon species, their sex, and their age. For example, chinook generally survive better than Atlantic salmon against *Chaetoceros*, but may suffer equally with *Heterosigma*. Mixed-sex coho do well against *Chaetoceros* and *Heterosigma*, whereas sterile coho are hardest hit. Two-year-old Yukon/Big Qualicum chinook hybrids were harder hit (93%

mortality) by the 1997 *Heterosigma* bloom than the regular chinook stock (45% mortality). Local fish species can accommodate best to local phytoplankton species. These observations show the importance of the parental stock. Mature fish are generally the most sensitive of all, likely to result in total mortality due to *Heterosigma*. Sometimes stocking the cages with a low number of fish can protect the fish, but this is not always successful.

With respect to the algal species, *Chaetoceros* generally shows a consistent mortality above a threshold cell number (5000 – 6000 cells L^{-1}), although fish stocks become more sensitive during the summer when temperatures are high and dissolved oxygen is low. In contrast, there is a non-linearity between *Heterosigma* cell numbers and fish mortality. This may reflect the different cellular toxicities, as well as temperature effects.

2) Protocols aimed at mitigating the adverse effects of HABs

Rensel (1995) originally wrote a chapter on managing finfish aquaculture resources, which includes mitigation factors at finfish aquaculture sites, for the IOC Manual on Harmful Marine Algae. The continued seriousness of the problem has now resulted in a much expanded and revised manuscript, by Rensel and Whyte, to be included in the next edition of the IOC Manual. The following summary was given of the major methods to mitigate adverse effects of HABs on fish farming, based on material presented from the revised IOC chapter plus the panel and general discussions:

- Airlift pumping
  - Effective only if the HAB is restricted to the surface
  - Works for *Heterosigma*, but not for *Cochlodinium*, which can be found even at depth
- Oxygenation and aeration
  - Not the best approach, because of the adverse effects on fish physiology
  - If used, employ skirts to contain the water
- Moving net pens
  - Risky, because of possible structural damage to pens, crowding of fish in nets causing stress to fish
  - It is not always clear where to tow the pens, and can interfere with shipping
- Submerging net pens
  - Feasible if sufficient water depth is available
- Alternative culture systems
  - Consider new cage designs, offshore pens, enclosed ocean pens, onshore pens
  - Often an expensive solution, and must deal with waste disposal
- Feeding and handling practices
  - Stop feeding the fish and eliminate unnecessary noise and traffic near pens
- Therapeutics
  - None are readily available or government approved; costly to develop
  - Those designed to remove gill mucus may lead to secondary problems due to mucus loss over the whole fish

- Monitoring
  - Understanding seasonality of blooms in order to predict HABs
  - Using satellite imagery and remote sensing to track HABs
  - Train farm personnel to identify HAB species
- Chemical control
  - Apply copper sulfate, potassium permanganate, zinc compounds, hydrogen peroxide chlorine, or other algaecides
  - Flocculate cells from the water column by using clay or alum, as long as it can be quickly swept away and if net pens are far from shellfish aquaculture sites
- Biological control
  - Polyculture: grow bivalves within or surrounding pens; AquaNet funding being used to study mussel/kelp/salmon culture in the Bay of Fundy; are theraputants taken up by the polycultured organisms?
  - Zooplankton: rotifers, ciliates, copepods; production of grazers impractical
  - Bacteria: *Cytophaga* sp. isolated from *Chattonella*, other algicidal bacteria
  - Parasites: *Amoebophyra ceratii* parasite of dinoflagellates, but generally non-specific
  - Viruses: *Heterosigma* virus (rapid growth and host specific)
- Other methods
  - Improve environmental conditions (e.g. by decreasing nutrient output, rehabilitate and restore waterways) in order to discourage formation of HABs
  - Improve site classification and selection criteria (choose locations that are well mixed, in upwelling, frontal or estuarine zones)

RECOMMENDATIONS

- Improve current practices of depuration, pen design, skirt design, bubbling technologies, towing methods, and husbandry practices.

- Continue to develop physical, chemical, and biological factors to control and mitigate the harmful effects of HABs. In particular, research on viruses specific to certain HAB species was highlighted as a promising avenue of research, because of their specificity (use a cocktail of viruses), high titer (only a small amount needed), and short half-life in seawater.

- Improve monitoring approaches by investigating alternate sentinel species, training farm personnel to identify HAB species, and taking advantage of remote sensing technologies.

- Build up databases of recurring HAB occurrences, in order to avoid those locations during the selection of future aquaculture sites. Ensure the quality control of the data. Salvage existing databases that have not yet been placed in an electronic form. If collected as part of a research program, make the data public after a two-year period. Integrate such databases into GIS programs.

- Improve existing and develop new field "dip stick" methods for phycotoxin detection, in order that aquaculturists may screen their product.

- Promote better communication among academics, federal and provincial agencies, and fishing and aquaculture industries in order to warn of the presence of HABs at aquaculture sites. The telephone and the Internet can be better employed in this regard.

- Incorporate aquaculture research findings from other countries into plans for the development of Canadian aquaculture. Integrate HAB research with coastal planning.

- Carry out research to generate information that would address public opposition to existing and planned aquaculture sites, as well to the use of certain biological and chemical controls.

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REPORT OF THE WORKING GROUP B

PHYTOPLANKTON MONITORING RELATED TO TOXIN DISTRIBUTION, AND INNOVATIONS AND APPLICATION OF BIOTOXIN ASSESSMENT RELATED TO PUBLIC HEALTH ISSUES

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INTRODUCTION

Harmful algal blooms (HABs) and the phycotoxins they produce represent a significant and seemingly expanding threat to human health, fishery resources, and marine ecosystems throughout the world. Of major concern to human health are the HAB species that cause paralytic, amnesic, diarrhetic, neurotoxic, and azaspiracid shellfish poisoning (PSP, ASP, DSP, NSP, and AZP), and ciguatera fish poisoning. In most countries, the mouse bioassay and/or chemical assays are used to detect toxicity in shellfish and finfish tissues for regulatory purposes. In addition, many countries maintain HAB monitoring programs as an early warning system to minimize the consequences to marine resources and human health.

The mouse bioassay (AOAC International, 1995) is used in most countries to screen shellfish for the presence of PSP toxins. Although the mouse bioassay has been very effective at protecting the public from shellfish contamination for past decades, the use of live animals presents ethical problems and several participants at the workshop stressed the need for a safe, reliable way to replace the mouse bioassay. Furthermore, improvements in biochemical assays and instrumental analytical technologies are continuously being made in the field of phycotoxin analysis.

The questions addressed in this workshop were 1) What is the future of the mouse bioassay and what are potential alternatives? 2) What is the position of regulatory agencies? and 3) What is the future of HAB monitoring programs? Following is a summary of the discussion.

1) What is the future of the mouse bioassay and what are potential alternatives?

In order to assure a continued, safe supply of seafood products, it is necessary to continually screen shellfish for toxicity in areas influenced by HABs. Of all the toxins produced by harmful algae, our knowledge is by far the most complete for PSP, primarily since it is a world-wide problem. The mouse bioassay is the official method for PSP analysis (AOAC International, 1995) and is currently used by most countries. In recent years, there has been much discussion on the merits and drawbacks of the mouse bioassay. Among its advantages are that it is relatively easy to perform, requires no special equipment, detects total toxicity, and gives reproducible results (Sullivan *et al.*, 1988). However, a number of disadvantages have been mentioned in the literature. The susceptibility of mice strains varies (McFarren, 1959), animals of uniform size are required (Shimizu, 1988), and a large supply of mice must be maintained (Sullivan *et al.*, 1988). Additionally, a protective "salt effect" may cause an underestimate of the toxin concentration at low toxin levels (Schantz *et al.*, 1958). This assay is limited in its minimum detection limit of approximately 37μg/100g meat, which is close to the maximum allowed level of 80μg/100g meat (Yang *et al.*, 1987). Since the relationship between dose and death time is logarithmic, this assay may require the use of as many as 8-12 mice to achieve the proper death times by trial-and-error dilutions (Sullivan *et al.*, 1988). Participants in the workshop emphasized that the use of live animals in experiments raises ethical questions and is especially of concern in the UK. For these and other reasons, efforts are being made to find an acceptable replacement.

Alternative methods for the detection of toxins (e.g. immunoassays, enzyme-based assays, molecular probes, and liquid chromatography) have been discussed for a number of years, yet few are implemented in countries conducting large-scale monitoring of their shellfish industries. The discussion of this working group focused principally on new rapid screening tests, instrumental analytical methods, and on the difficulty of obtaining AOAC approval for new tests to substitute the mouse bioassay.

The application of rapid screening tests was first discussed. An approach that has great potential appears to be the MIST Alert™ test (Jellett Biotek Ltd., Dartmouth, NS), a rapid immunodiagnostic test for the detection of marine biotoxins (PSP and ASP) in shellfish or phytoplankton. This type of test could be used for rapid screening and early warning. It could be especially useful in remote areas for community-based monitoring of harmful toxins. J. Jellett gave the example of PSP outbreaks on Kodiak Island, Alaska, which cause human sickness or death each year. On this Island, volunteers (teachers, nurses, children, etc.) run a monitoring program using the MIST Alert™ test. If positive results are detected, samples are then sent to regulatory agencies for confirmation by more conventional methods. This type of collaboration could increase the extent of the coastline that is currently monitored. With regards to site selection in the shellfish industry, rapid screening tests could provide broad-scale toxin mapping of coastal zone areas under consideration for the development of aquaculture sites.

Although new rapid assay methods appear to be very promising for the replacement of controversial animal assays as screening tools, these methods usually require confirmation of positive results by more sophisticated methods. Moreover, these assays are limited by their specificity. If several methods must be used to monitor all toxin classes, the workload of regulatory agencies will be greatly increased. There is thus a trend towards the development of

multi-toxin techniques, such as liquid chromatography-mass spectrometry (LC-MS) which can detect and identify many different toxins in one single analysis. However, although LC-MS works very well for certain toxins, the sensitivity still has to be improved for others. Even though the cost to run samples is decreasing, the LC-MS method still requires qualified personnel to properly run the instrument and ensure quality control.

Participants further indicated that there is currently a pressing need for certified calibration standards and reference materials. M. Quilliam noted that the NRC Certified Reference Materials Program (CRMP) requires additional resources to pursue the preparation of new materials. Steps involved in the preparation of a new material include the isolation and purification of individual toxins, detailed stability studies, packaging into flame-sealed ampoules, and high accuracy analytical measurements. A dedicated infrastructure is also required for the distribution, maintenance and replacement of products. All this is very expensive and the CRMP presently operates on a cost recovery basis. Recent funding from the Asia-Pacific Economic Cooperation (APEC) should partially alleviate this problem by providing seed money for new products.

A combination of techniques is probably the best approach to pursue, such as conducting screening tests to sort out the negative samples from the positive samples, thereby resulting in only a few samples to be run by conventional methods. This could reduce the overall cost.

2) What is the position of regulatory agencies?

Since the Canadian Food Inspection Agency (CFIA) will have to process an increasing number of samples in the near future, its capacity to analyze samples will also have to increase. From a regulatory standpoint, this will require not only rapid but also simple and cost-effective methods. It was suggested that a useful regulatory method for rapid toxin detection could be a simple immunoassay, such as the MIST Alert™. Although this method would be unable to give an absolute number for toxicity, it would allow regulators or harvesters to quickly know the toxic status of a sample. This type of screening test could reduce both costs and animal use in regulatory laboratories. However, it was noted that this method would not be able to detect the presence of new toxins that would otherwise be detected by traditional tests such as the mouse bioassay. Finally, participants pointed out the difficulty of obtaining AOAC approval for several of these new tests, and it was noted that there are alternate routes.

3) What is the future of HAB monitoring programs?

Most countries maintain harmful algal bloom (HAB) monitoring programs as an early warning system to minimize the negative consequences to marine resources and human health. Because these monitoring programs are based on the identification and enumeration of harmful algae by optical microscopy, they are often labor-intensive, time-consuming, and require extensive taxonomic training. In fact, participants emphasized the current need for more taxonomists and algal scientists in Canada.

Participants stressed the importance of maintaining HAB monitoring programs. First, since these programs examine the whole phytoplankton community, new and potentially harmful species are identified and enumerated. Second, these monitoring programs allow hindcasting. Examples of

how HAB monitoring programs may reinforce toxin monitoring programs have been shown for the Bay of Fundy and Gulf of St. Lawrence, where the sudden detection of domoic acid in shellfish was rapidly linked to *Pseudo-nitzschia pseudodelicatissima* and *P. seriata* blooms, respectively. Third, HAB monitoring programs, which are usually backed by basic environmental measurements, result in long time series data which allow for a better understanding of HAB bloom dynamics. For example, the analysis of the 10-year data set from the HAB monitoring program conducted in the St. Lawrence since 1989 revealed a close relationship between rainfall, local river run-off and *Alexandrium tamarense* blooms at a coastal monitoring station in the Gulf of St. Lawrence (Weise *et al.*, 2001). Finally, HAB monitoring programs are essential to properly assess the risk of introducing toxic species via ship ballast exchanges or shellfish transfers.

An integrated program involving harmful phytoplankton monitoring, combined with confirmatory testing of toxins in shellfish, was favored by most participants. Due to the complex hydrodynamic nature of coastal areas (tides, currents, etc.) and the sampling frequency of most phytoplankton monitoring programs (e.g. weekly), one may miss the presence of toxic cells in the water column and thus possible toxic shellfish events. Another point that was raised during the discussion was the case of benthic and epiphytic algae, which are missed by most current HAB monitoring programs that focus on plankton. Knowledge of toxin origin in a given area is thus necessary in developing an adequate monitoring program.

Current DNA probes that specifically bind to toxic species open the possibility for the development of automated, *in situ* sampling devices. An automated sampling device able to recognize and enumerate *Alexandrium* spp. cells is presently in the testing phase in the Gulf of Maine as part of the GOM-ECOHAB program. In areas of known recurrent toxic blooms, the use of such automated buoy systems offshore is certainly promising (e.g. the work of C. Scholin in Monterey Bay, California). However, these buoys can only complement existing conventional HAB monitoring programs since they are limited by the specificity of their probes.

Finally, the participants of the workshop proposed a multi-step approach. First, a HAB monitoring program should be in place as an early warning system. Second, rapid assays for toxin screening should be used, followed by the confirmation of toxins by more sophisticated chemical methods.

RECOMMENDATIONS

- Skilled taxonomists are needed. Several participants indicated that there is a shortage of personnel with extensive taxonomic experience. Training graduate students in algal taxonomy and ecology should be encouraged.

- There is an urgent need for certified toxin standards. Several participants stressed the importance for the NRC Certified Reference Materials Program to pursue the development of certified standards.

- Better communication among academics, federal and provincial agencies, and fishing and aquaculture industries should be promoted. Communication and collaboration between CFIA and DFO scientists, in particular, must be strengthened.

- Maintain established HAB monitoring programs along Canadian coastlines. The lack of comprehensive HAB monitoring programs on the West Coast, as well as in the southern Gulf of St. Lawrence, was often mentioned.

- Community-based monitoring, especially in remote areas and on First Nations territories, should be promoted. This could be in the form of using rapid screening tests. If positives are obtained, samples should be confirmed by regulatory agencies.

- The participants of the workshop proposed the use of a combination of methods in monitoring programs to ensure public health and minimize the consequences to marine resources:
  - Early warning toxic phytoplankton monitoring
  - A combination of techniques for the detection of toxins, e.g. rapid low-cost assays to screen for negatives, and confirmation of positives by HPLC and LC-MS.

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